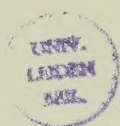


PARAMETERS OF PROGNOSIS IN ACUTE MYELOID LEUKEMIA

**Evaluation of growth pattern in agar,
karyotype and in vitro sensitivity to
chemotherapy agents**

Anita Gustavsson

Lund 1984



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Abstract The prognostic value of in vitro growth pattern, cytogenetic pattern and in vitro drug sensitivity in AML was studied. Patients with growth of colonies (A) or a normal number of clusters without colonies (B) had significantly higher remission rate than patients with an excessive number of clusters (C) or no growth in vitro (D). In ALL the growth pattern had no prognostic value. An association between cytogenetic pattern and growth pattern was found in AML. Patients with normal metaphases (NN) mostly showed favorable growth pattern (A+B) while patients with abnormal metaphases only (AA) predominated in the unfavorable growth patterns (C+D). Combination of growth pattern and cytogenetic pattern did not improve the prognostic judgement. The in vitro sensitivity of clonogenic cells to ara-C and DNR in AML showed that patients achieving remission had significantly higher sensitivity to both drugs than patients not entering remission. Serial studies of in vitro growth during the course of AML showed oscillations in the number of clusters during induction of remission with a short phase of no growth preceding remission. During remission the growth showed normal features and at the time of relapse growth decreased or ceased. The percentage of blast-like cells in S-phase was significantly smaller than that of cluster-forming cells. A comparison of the total number of cluster-forming cells and blast-like cells in S-phase suggested nonidentity of the two cell populations.			
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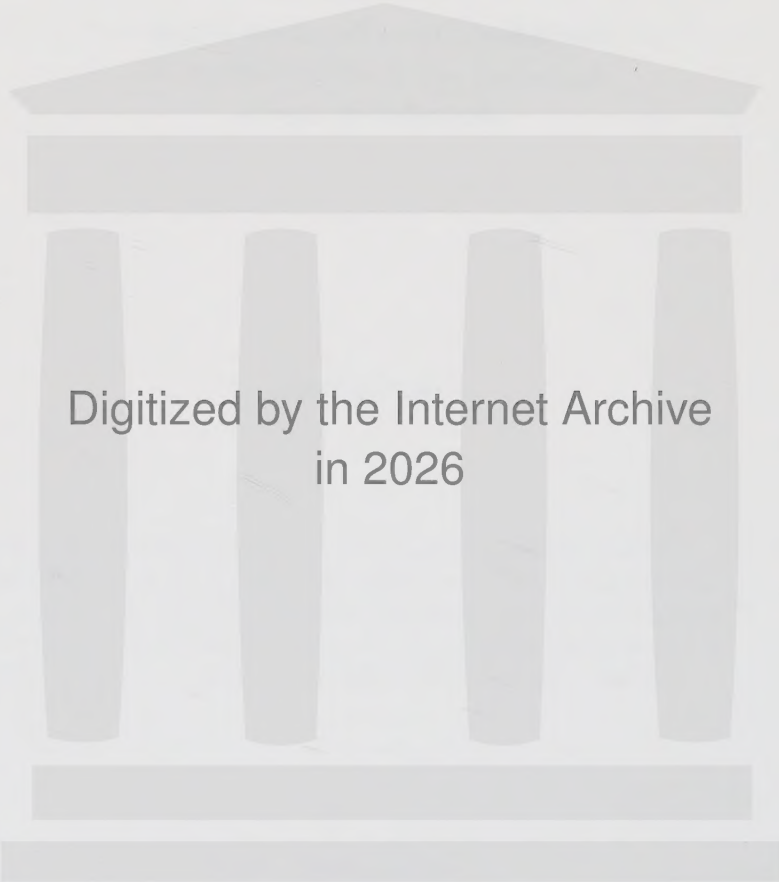
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Hematology, University of Lund, Sweden

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This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

- I. Gustavsson A, Olofsson T, Olsson I & Sigurdardottir H.
The prognostic significance of in vitro bone marrow growth pattern in acute non-lymphocytic leukaemia.
Scand J Haematol 26, 364-377, 1981.
- II. Gustavsson A, Mitelman F, Olofsson T & Olsson I.
The relationship between growth in agar, karyotype and prognosis in acute leukaemia.
Scand J Haematol. In press.
- III. Gustavsson A & Olofsson T.
Prediction of response to chemotherapy in acute leukemia by in vitro drug sensitivity testing on leukemic stem cells.
Submitted for publication.
- IV. Gustavsson A, Olofsson T & Olsson I.
Oscillations of marrow culture growth in acute myeloid leukemia during remission induction and remission.
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- V. Gustavsson A, Olofsson T & Olsson I.
Relationship between myeloblasts and cluster-forming cells in acute non lymphocytic leukemia.
Hematol Oncol. In press.

ABBREVIATIONS

ANNL	acute non lymphocytic leukemia
AML	acute myeloid leukemia
M 1-6	French-American-British classification of AML
ALL	acute lymphocytic leukemia
CFU-S	colony forming unit in spleen
CFU-D	colony forming unit in diffusion chamber
CFU-GM	colony forming unit - granulocytes, macrophages
CFU-C	colony forming unit in culture (= CFU-GM)
BFU-E	burst forming unit - erythroid
CFU-E	colony forming unit - erythroid
CSA	colony stimulating activity
GM-CSF	granulocyte, macrophage-colony stimulating factor
HP-CM	human placenta-conditioned medium
PHA-LCM	phytohemagglutinin - leukocyte conditioned medium
³ H-TdR	tritiated thymidine
LI	labeling index
SI _{C1}	suicide index for clonogenic cells
Ara-C	cytosine arabinoside (Cytosar ^R)
DNR	daunorubicin = rubidomycin (Cerubidin ^R)
TRAP	thioguanine, rubidomycin, ara-C, prednisone
COAP	cyclophosphamide, Oncovin, ara-C, prednisone
NN, AN, AA	cytogenetic patterns; NN only normal metaphases, AN abnormal and normal metaphases, AA only abnormal metaphases
LD ₅₀	the drug concentration causing 50 per cent reduction of clonogenic cells

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INTRODUCTION

In 1845 leukemia was recognized as a clinical entity in descriptions by Craigie (44), Bennett (12) and Virchow (207). Two years later Virchow coined the term "leukemia" proposing that there was an increased number of the colourless cells that normally occurred in the blood. At that time the origin of blood cells was unknown. In 1870 Neumann demonstrated from autopsy findings that the bone marrow was an important site for the formation of blood corpuscles (155). The introduction by Ehrlich of staining methods made it possible to recognize various forms of leukocytes and in 1900 Naegeli described the myeloblast (153) and distinguished between lymphoblastic and myeloblastic leukemia. In 1913 Reschad and Schilling-Torgau described monocytic leukemia (177) and four years later Di Guglielmo recognized malignant proliferation of erythrocytic precursors (52).

One of the most important goals in the management of acute leukemia is to define parameters of prognosis so that the chemotherapy treatment can be individualized. Methods for growing normal and leukemic progenitor cells in vitro has provided new possibilities for that purpose. The major aim of this thesis is therefore to investigate if such methods can be applied for prediction of prognosis in the treatment of acute leukemia.

TREATMENT MODALITIES IN ACUTE LEUKEMIA

Before the use of chemotherapy, patients with acute leukemia succumbed in bleedings or infections with a median survival of 1-2 months (122, 201). Spontaneous remissions occurred rarely but if so with a median duration only of 2-4 months, even if exceptional patients survived for about 4 years (190, 201).

Successful treatment of leukemia started with the use of a folic acid antagonist, aminopterin, which induced temporary remissions in children with ALL (60). A combination of aminopterin and cortisone was utilized in 1950 in childhood ALL with promising results (17). Combination chemotherapy has subsequently led to long term remission (>5 years) of more than half of all children with this disease (140).

Single drugs effective in ALL gave remission rates in AML below 20 per cent (71). Multiagent therapy was therefore used based on the idea that the antileukemic effects of different agents would be additive or synergistic. The POMP combination (prednisone, Oncovin, methotrexate and Purinethol) improved remission frequency in AML to about 30 per cent (179). A new drug, cytosine arabinoside (ara-C) gave a response rate of about 25 per cent (58) and in combination with thioguanine approximately 40 per cent (36, 120). Daunorubicin, an anthracycline antibiotic, is the hitherto most effective single drug in AML with remissions in the range of 50 per cent (19, 211). The observation that severe bone marrow hypoplasia was essential to achieve remission resulted in the use of intensive chemotherapy aiming at destruction of all leukemic cells. Normal hemopoiesis is supposed to recover more rapidly than the leukemic clone, even if the therapeutic index is very narrow. Current therapy regimens consist of combinations of anthracycline, ara-C, thioguanine and/or vincristine with or without prednisone giving 60-80 per cent remission rates (70, 76, 124, 162, 167, 175, 203) even in patients above 60 years of age (66, 167, 176). Median duration of remissions is 9-19 months and has not improved substantially in recent years (72). Only 10-20 per cent of the patients have a probability of long term survival (72). The therapeutic value of consolidation therapy, maintenance therapy, and late

intensification to eradicate remaining leukemic cells and prolong the duration of remission, can not yet be evaluated. Post remission therapy is obviously necessary but its design is not clarified.

Bone marrow transplantation is another established treatment of AML when an HLA-identical sibling is available as a donor (161). The 2 year survival for patients aged 30-50 years is about 30 per cent while it is 70 per cent for patients below 20 years of age. (199). The value of transplantation is presently being compared to the results achieved with intensive chemotherapy alone with possible cure of an increasing number of patients (53, 73).

HEMOPOIETIC STEM CELLS

Hemopoietic tissue contains pluripotent stem cells capable of extensive proliferation and commitment to differentiation along a hematopoietic lineage. Differentiation leads to an altered phenotype with development of the physiological functions of the mature cells.

In vivo and in vitro clonogenic assays for stem cells are now available. Bone marrow cells injected into lethally irradiated mice produce colonies in the spleen (200) from single cells as shown by chromosomal analysis (9). The colonies consist of erythroid, granulocytic, megakaryocytic and undifferentiated cells (67, 119) demonstrating their origin in a pluripotent stem cell. The operational term for the multipotent stem cell is "colony-forming unit in spleen" - CFU-S. The CFU-S has self-renewal capacity as evidenced upon serial transplantation (186). Chromosome marker studies indicate that hemopoietic stem cells give rise to both myeloid and lymphoid cells (2). In steady state less than 10 per cent of CFU-S appear to be in S-phase as indicated by "suicide" experiments with ^3H -TdR causing death of cells in DNA-synthesis (10, 115). The proliferative state of CFU-S is influenced by the concentration of CFU-S in the marrow; when the hemopoietic tissue is partially depleted by irradiation the remaining CFU-S proliferate at an increased rate (38). The self-renewal potential of CFU-S appears to exceed the life-span of the individual even if a decline can be demonstrated by serial trans-



plantation (187). Alkylating agents decrease the self-renewal capacity of murine CFU-S (20, 21), which may explain late sequelae in chemotherapy.

Murine (105, 139) and human (8, 61, 133) pluripotent stem cells can be cultured in agar or methyl cellulose with the production of mixed colonies with erythrocytes, neutrophils, macrophages, eosinophils and megakaryocytes, and both murine (49, 50) and human (74) hemopoietic stem cells can be grown in long term culture on an adherent cell layer. The progenitor cells for granulocytes and macrophages (CFU-GM) detected by agar culture are described below. Granulocytic precursor cells (CFU-D) can also grow in diffusion chambers implanted into the peritoneal cavity of mice (11, 35). Such precursor cells may represent more immature cells than CFU-GM (101, 158). Culture methods have also been developed for progenitors of erythropoiesis (BFU-E and CFU-E) (89, 196), megakaryopoiesis (136, 154) and eosinophilopoiesis (135) as well as for T and B lymphocytes (63, 137, 195).

STEM CELL ORIGIN OF LEUKEMIA

AML can originate in a pluripotent stem cell which is supported by the finding of identical chromosomal aberrations in both myeloid and erythroid precursors (18). The distribution of the X-chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) also indicates an origin in a pluripotent cell (62).

Development of AML is a multi-step process, where perhaps the transformed stem cells initially follow normal differentiation programs. After a long latency period secondary events lead to proliferation of leukemic cells with a block in differentiation. Cases with AML preceded by a clinically recognizable preleukemic state suggest such a multi-step mechanism. Because of a growth advantage for leukemic cells normal progenitor cells are suppressed, but when remission occurs normal hemopoiesis again repopulates the marrow.

The accumulation of leukemic blast cells is assumed to depend on a block in cell differentiation (182). According to one concept leukemic cells are thought to be "frozen" at a normally transient

stage of cell differentiation. Another hypothesis suggests that the leukemic blast cells represent a separate lineage not directly related to normal differentiation (126). The findings of lineage infidelity in blast cells, both intramyeloid (the individual cells contain markers of more than one myeloid lineage) and interlineage (markers of both myelopoiesis and lymphopoiesis) (189) do not support the view of a block at a normally transient stage of differentiation.

AGAR CULTURE GROWTH OF GRANULOCYTE-MACROPHAGE PROGENITORS (CFU-GM)

Culture of CFU-GM (22, 168, 169) has been of profound importance for studies of regulation of human hemopoiesis, both normally and in leukemia. CFU-GM is a precursor cell committed for the granulocyte-monocyte-macrophage pathway of differentiation, and produces colonies arising from single cells (145) and consisting of granulocytes and/or macrophages (55). The majority of CFU-GM are in active cell cycle with 30-45 per cent in S-phase as judged by ^3H -TdR-suiciding (85, 98, 115, 147, 159). The capacity for self-renewal is, however, limited (138). The CFU-GM compartment is not completely homogeneous. Thus separation of marrow cells in velocity sedimentation has shown not only non-identity with CFU-S (212) but also two classes of CFU-GM with peaks of colonies on day 14 and day 7 of culture respectively (101, 104).

The production of colonies is dependent on the addition of a growth factor, CSA. GM-CSF is the operational name given to the CSA which stimulates proliferation and differentiation of granulocyte-macrophage progenitors. Human GM-CSF is produced by a number of different cell types; monocytes (77), PHA-stimulated T-lymphocytes (42), endothelial cells (111, 172), bone marrow adherent cells (184), placental trophoblasts (181), some human T-lymphocyte cell lines (79), and a human monocyte-like cell line (54). Several tumors can produce CSA e.g. a pancreatic tumor (213), a soft tissue sarcoma (94), Hodgkin cell lines (33) and lung cancer (7, 109).

CSA from human placenta-conditioned medium (HP-CM) has been extensively purified and found to be a glycoprotein with a Mw of about 30,000 (30, 156). Sub-classes of GM-CSF have been identified; GM-CSF (α) preferentially stimulates CFU-GM day 14 with colonies consisting

of granulocytes and macrophages, and GM-CSF (β) preferentially stimulates CFU-GM day 7 with colonies primarily consisting of neutrophils; in addition an Eos-CSF has been identified (156). At low concentration, purified GM-CSF favors macrophage production, whereas high GM-CSF concentrations favor neutrophil production (156). With increasing concentrations of CSA more non-cycling GM-precursors enter S-phase and the lag phase before growth in agar begins is shortened as well as the doubling time for the clonogenic cells and the colony size enlarges (32). CSF also shows some stimulatory effects on mature neutrophils and macrophages (31).

CSA is obligatory for growth of CFU-GM in vitro. Cyclic fluctuations in urine and plasma in cyclic neutropenia (123, 149) suggest a role in vivo of CSA. This is also suggested by the implantation of a human CSA-producing tumor in nude mice causing marked neutrophilia which was reversed by resection of the tumor (7).

The proliferation of CFU-GM begins asynchronously after a short lag period and the number of cell aggregates rises linearly to a maximum at 4-7 days consisting mainly of clusters of 3-40 cells. Thereafter some aggregates disappear but some continue to grow to form colonies of more than 40 cells. On day 7 cluster to colony ratio is 5-10:1. The number of colonies peaks at day 8-12. During the culture period the cells in colonies and clusters differentiate to mature neutrophils and macrophages. Both mixed colonies and colonies consisting of only one cell type occur and the cell composition changes during the growth period with an early dominance of neutrophils.

Agar culture of CFU-GM has led to the identification not only of growth stimulatory factors but also of inhibitory factors. Prostaglandin E, produced e.g. by macrophages, inhibits the growth of CFU-GM (165, 166). Acidic isoferritins derived from monocytes-macrophages inhibit CFU-GM in S-phase (26, 27) and iron-saturated lactoferrin from mature neutrophils inhibits CSA-production from monocytes and macrophages in vitro (24, 25). A factor called Leukemia Associated Inhibitor (LAI) inhibits CFU-GM proliferation (160).

Serum and plasma lipoproteins inhibitory for the growth of CFU-GM have been described (14, 37, 83). In addition, subpopulations of peripheral blood cells (193, 194) and NK-cells (92) show inhibitory activities towards CFU-GM. It is not clear if the above mentioned inhibitors have a physiological role in the regulation of granulopoiesis.

IN VITRO CULTURE OF LEUKEMIC CELLS

The agar culture system has been used extensively for studies of cells from patients with AML. An abnormal growth pattern has been found mostly without colony formation but with growth of various numbers of clusters or no growth at all (84, 147, 150). The growth pattern does not correlate to the different morphologic subtypes of AML. Leukemic clonogenic cells can be distinguished from normal CFU-GM by their low density (147). Leukemic cells require GM-CSF for in vitro proliferation (99, 134) and a decreased sensitivity of leukemic cells to GM-CSF has been reported (68). The CSA production in leukemia is low (69, 78, 84, 88), normal (148), or high (80, 150).

The leukemic origin of colonies and clusters is based on the finding of chromosomal markers for leukemia (56, 146, 202), and defective maturation with bizarre forms (110, 112, 147, 150, 191, 206).

During remission the growth pattern becomes normal. In relapse the abnormalities reappear. However, it must be emphasized that agar culture supports growth of both normal and malignant myeloid cells and it is always difficult to decide if individual clusters or colonies have a normal or leukemic origin.

Methods for selective growth of leukemic cells on stimulation with PHA or PHA-LCM have been reported (28, 51). When blood cells from patients with AML are cultured in methyl cellulose supported by PHA-LCM (28), colonies with cells of blast like morphology and leukemic chromosomal markers develop (100, 202). These cells have a high S-phase fraction (141) and capacity for self-renewal. Such cells have been suggested to represent an abnormal population not present within the normal myelopoietic lineage (129) which by its high proliferative activity can sustain the leukemic cell production (141).

PROGNOSTIC EVALUATIONS IN ACUTE LEUKEMIA

a) Predictive value of various factors

Age is related both to response rate to therapy and survival. Advanced age has been implied to correlate with a poor prognosis because of failure to tolerate intensive chemotherapy rather than drug resistance (59). Some claim, however, that with adequate supportive care remissions in elderly patients are as likely to occur as in younger patients and lasting as long (82), but this is not an unanimous opinion (102, 107).

Leukemia developed in a patient with a preleukemic syndrome as well as after treatment with radiation and/or alkylating agents for other malignancies has a bad prognosis (43, 107, 121).

High WBC and/or low platelet counts, earlier recognized as predictive factors, seem to be of less prognostic value today because of improved supportive care. The presence of fever at the time of diagnosis is a bad prognostic sign in some (107) but not all reports (102).

Significant differences in prognosis between morphologic subgroups of AML (13) have not been unequivocally shown except for the long duration of remissions in promyelocytic leukemia (125, 210). The presence of Auer rods in blast cells may indicate a favorable prognosis (107, 131).

Prognostic evaluations of cell cycle activity of leukemic cells are contradictory. In several studies no correlation was found between ^3H -TdR LI and remission rate (5, 45, 116, 205, 208) whereas a high LI signified a high frequency of remission in others (93, 215). Agreement exists, however, that a high LI implies a short duration of remission (45, 93, 208).

Predictive models of prognosis using multivariate logistic regression analysis (188) showed long remissions in patients with low lactate dehydrogenase, low or normal fibrinogen level, a single course of chemotherapy to achieve complete remission and a rapid disappearance of leukemic cells in blood (125). These analyses could help to detect

patients at a high risk of relapse and might be useful in designing therapeutic strategies to improve duration of remission. However, the predictive variables may differ between various treatment regimens.

One major undisputable prognostic factor for long survival is the achievement of complete remission.

b) Predictive value of in vitro cultures

Several studies before and during the present investigation have been performed to ascertain the prognostic value of in vitro culture of bone marrow or of blood cells from AML patients. Reports of bone marrow cultures at the time of diagnosis are summarized in table 1. Direct comparison of data is obviously difficult because of differences in chemotherapy treatment and in methodology, e.g. scoring time for the growth pattern. In spite of this in most studies the in vitro growth patterns has been found to be helpful in predicting the success or failure of remission induction therapy. Failure to grow or decreased growth in agar cultures of marrow cells was most often a favorable prognostic sign (16, 39, 103, 110, 112, 191, 206). In a few reports, however, no growth was found to be an unfavorable sign (132, 150, 178). Prognostic implications from the growth pattern of blood cells have also been reported (16, 103, 178, 206).

The growth pattern in vitro at diagnosis does not seem to predict duration of remission or survival time (39, 110, 112, 132, 178). In one study, however, patients with growth of small clusters only had longer remissions than patients with other growth patterns (106). AML patients with detectable clonogenic cells in blood have longer survival than patients without (96); an increased number of such cells signifies a long duration of remission (103). Moreover, low self-renewal of blast progenitor cells in blood signified a high remission rate and long survival (128).

The in vitro growth pattern appears to be independent of variables such as age, leukocyte and platelet counts or infection status and seems to be a separate prognostic factor (191).

Table I. In vitro growth pattern in bone marrow cultures from patients with AML

Reference	No pts	No of therapies	Remission rate (%)	Incubation days	Definitions cluster	Definitions colony	Comparison between growth pattern and remissions No CR/total number	Col. + cl.	Clusters	No growth
150Moore et al. 1974	108 ¹ (83 pts treated)	6	36 (46 pts treated)	7	>3	>40	(3-40 cells) 3/26 7/18* 27/51*	(3-20 cells)	2/13	
39Chervenick, 1975	33	2	42	14	>10	>50	4/10	1/6 ²	9/17* ³	
178Robinson et al. 1975	19	2	26	14		>50	5/10*		0/9	
206Vincent et al. 1977	38	2	42	14	>3	>50	3/12	0/4	13/22*	
132Mertelsmann et al. 1981	96	2	59	7	>3	>40	7/16	40/55* ⁴	10/25	
96Hörnsten et al. 1982	45	4	40	14	-	>20	10/31(>1 col)		8/14	
46Curtis et al. 1975	42	1	46 (PR+CR; 52)	14	-	>20	6/13(>31 col)	8/14(3-30 col)	8/15(<3 col)	
191Spitzer et al. 1976	58	1	59	7	>3	>40	(21-40 cells) ⁵ 4/17 26/35* 4/6* ⁶	(3-20 cells)		
112Knudtzon,	30	9	53	7	>5	>50	(>1000 cl) 3/10 (101-1000 cl) 4/8*	(0-100 cl)	9/12	

24	2	54	>8	>50	1/10 "excessive growth"	12/24* "low growth"
10 ³ Kirshner et al. 1982	30	1	60	14	1/4 (>50 col) A 9/7 (col+>50 cl)	6/7 (no growth) B*4/4 (<50 col or cl) 7/8 (only cl)
10 ³ Jehn et al. 1983	31 ⁸	1	48	10	>3	>50
					high growth	low growth*

No pts; bone marrow from AML patients at the time of diagnosis which later received treatment and were evaluable. Remission rate; complete remission (CR) if not otherwise stated, PR, partial remission. Col; colony, cl; cluster. In all studies buffy coats are used but in Hörnsten's and Jehn's studies where 1.077 g/ml separated cells were cultured. Mostly data are given per 10⁵ seeded cells, but Knudtson, Vincent and Hörnsten plate 2x10⁵ cells and Jehn's data are per ml bone marrow or blood, * denotes the investigator's judgement of favorable growth pattern in terms of high remission rate.

1. Among the 108 patients are 15% pts with secondary leukemia or earlier preleukemia and 2 pts with smoldering leukemia and in some patients data are only available from blood cultures, but it is not stated in how many cases.
2. Only including when >50 clusters/plate.
3. Including cultures with clusters of 5-10 cells and <50 clusters/10⁵ cells.
4. Including <10 colonies with ratio col/cl, <1:30, also including 3 pts with high cloning efficiency, but a ratio of <1:30.
5. Including 6 pts with colony formation and abnormal ratio.
6. Including low incidence of colonies and clusters with a normal ratio (>1/17).
7. "Excessive growth"; increased number of clones mostly predominated by large clusters (20-50 cells). "low growth"; no growth or decreased number of clones compared to normal controls.
8. Including 7 patients with secondary leukemia or previous hematological disease.

In ALL bone marrow cells usually show low or no growth in agar (46, 57, 112, 132, 150, 152, 173, 191) without any prognostic implications (112, 132, 152).

Regardless of the pretreatment findings, the growth pattern in agar usually becomes normal during remission and fails to do so in patients who are unresponsive to chemotherapy. Reemergence of normal culture findings generally precedes clinical and morphological evidence of remission (151, 192) or coincides with remission (151). Normal growth pattern in agar cultures during the hypoplastic phase of treatment suggests complete remission. Onset of relapse can also be predicted early by detection of qualitative changes in growth pattern such as defective maturation in the cell aggregates or an abnormal ratio between clusters and colonies and an increasing fraction of clonogenic cells with abnormal low density (151). Variations in the number of colonies occur spontaneously during the remission phase (132, 151, 206).

A good correlation was reported between the production of CSA from adherent bone marrow cells and remission frequency (88). A decrease in CSA production from such cells during remission indicated that relapse soon followed. In another report no correlation was found between response to treatment and the capacity of blood or bone marrow cells to produce CSA (16), whereas one study showed that absence of CSA producing cells in peripheral blood implied a poor response to treatment and a short survival and vice versa (96).

c) Predictive value of chromosome abnormalities

About 50 per cent of patients with acute leukemia exhibit numerical and/or structural chromosome abnormalities in direct preparations using chromosome banding techniques (64, 143, 198) and almost all patients with secondary leukemia have chromosome aberrations (180). Short term cultures with or without methotrexate cell synchronization leading to elongated chromosomes where additional abnormalities could be detected, have suggested that almost all patients with leukemia may have some kind of karyotypic change (113, 214). Although, the chromosomal abnormalities are variable there is a striking tendency for a non-random involvement with certain chromosomes engaged in an

exceptionally high frequency. In adults with AML chromosomes 5, 7, 8 and 21 are most commonly engaged, either alone or in combinations (143). The translocation between the long arms of Nos 15 and 17, t(15;17) is only encountered in promyelocytic leukemia and t(8;21) with or without the loss of a sex chromosome is associated with the M2 subgroup (185). In ALL the chromosomes 1, 6, 8, 9, 14 and 21 are most often affected (143). The Philadelphia chromosome t(9;22) which is found in 90 per cent of CML occurs both in AML (1, 90) and ALL (198). Serial studies of AML patients have shown that during complete remission bone marrow cells usually have normal karyotype and with relapse the original abnormality reappears sometimes with evolution of additional clonal aberrations (91, 197).

In many reports a correlation has been shown between karyotype and survival in AML. Early work (183) showed patients with only abnormal metaphases (AA) in the marrow cells to have one third as long survival as those with only normal metaphases (NN). Patients with both abnormal and normal metaphases (AN) had an intermediate survival time. These observations have later been confirmed (4, 95, 157). However, conflicting results have been reported; some (64, 81, 97) reported that the karyotype was a prognostic indicator in typical AML but not in its subgroup monocytic leukemia while others (118) came to the reverse conclusion. A lack of prognostic implications of the karyotype pattern has also been reported (65, 91). In adult ALL patients the longest survival and highest remission rate were seen when all metaphases were normal or the modal number was greater than 50 chromosomes (198).

d) Predictive value of in vitro sensitivity to chemotherapy agents

In attempts to develop drug sensitivity tests for leukemia cells the effects of drugs on incorporation of labeled uridine or thymidine (41, 174, 215) or the effect on trypan blue exclusion capacity has been measured (117). Some correlation between in vitro results and clinical response was noted. However, in these reports the sensitivity of the entire population of leukemic cells was studied and the results may not be valid for the clonogenic progenitor cells, which supposedly are responsible for maintenance of leukemic blast cells (130). Studies on the in vitro effects of chemotherapeutic drugs (171) showed that

patients whose clonogenic cells were sensitive to both cytosine arabinoside and daunorubicin in vitro entered remission while patients resistant to one or both drugs failed to achieve remission. Another study (163) showed that a higher than normal sensitivity to drugs of the leukemic cells correlated with achievement of remission. The degree of in vitro sensitivity to ara-C but not to doxorubicin showed a correlation to successful induction of remission in another study (128). Leukemic clonogenic cells show a great variation in drug sensitivity compared to normal clonogenic cells (29, 87, 114, 163).

AIMS OF THE THESIS

When the present investigation was initiated, an increasing knowledge was emerging about the regulation of hemopoiesis based on studies with new techniques for growing hemopoietic stem cells in vitro reviewed above. These techniques were applied by several investigators also to leukemia demonstrating abnormal in vitro growth patterns of marrow cells. A major goal in the care of patients with leukemia is to define parameters of prognosis so that each patient can have the most optimal therapy available. This is the major goal of my thesis too. Therefore, the following questions have been asked:

1. Does the growth pattern in agar of marrow cells from patients with leukemia have a predictive value for the result of chemotherapy?
2. Can the prognostic judgements in leukemia be improved by combining the analyses of growth pattern in agar and of the karyotype? Is there a correlation between the growth pattern and karyotype in such a way that a specific growth pattern is associated with specific chromosome abnormalities?
3. Is it possible to assay in vitro the effects of chemotherapy agents on clonogenic cells from patients with leukemia and correlate the results to the in vivo sensitivity to these agents and the clinical outcome?

The work has been extended to additional problems related to the understanding of remission induction and relapse in leukemia and the following questions were also asked:

4. How does the growth pattern in agar of marrow cells from patients with leukemia change during induction of remission, remission and relapse? Do the findings explain how remission and relapse can occur?

5. What is the relationship, if any, in leukemia between in vitro clonogenic cells and morphologically identified blast cells? Are the clonogenic cells precursor cells for the blast cells?

THE PROGNOSTIC SIGNIFICANCE OF IN VITRO BONE MARROW GROWTH PATTERN IN ACUTE NON-LYMPHOCYTIC LEUKAEMIA (I)

The growth pattern in agar of bone marrow cells of 56 untreated patients with ANLL was studied. Principally two different therapy regimens were used: TRAP or prednimustine (chlorambucil-prednisone) with or without vincristine. Complete remission was defined as normal bone marrow cellularity with less than 5% blast cells, return of neutrophil counts to $1.5 \times 10^9/l$ or greater and platelet counts to at least $100 \times 10^9/l$. The frequency of remission was 26/56 (46%) in all patients and 22/33 (67%) in patients treated with TRAP. The double agar layer culture technique (168) was used and the cells were separated on Isopaque-Ficoll (34). Blood mononuclear cells obtained from one of two normal donors with about the same feeder capacity were used as a source of CSA. The seeding of 10^5 cells was found to be optimal. Cultures were scored on day 7 for small clusters (3-20 cells), large clusters (21-40 cells) and colonies (> 40 cells). Large clusters and colonies were also counted on day 10, when the number of colonies had reached their maximum.

Four different growth patterns were recognized; group A, colony and cluster formation; group B, growth of small clusters only but less than $600/10^5$ seeded cells; group C, growth of small clusters only but more than $600/10^5$ cells and group D, no growth in vitro. A cut off point at 600 small clusters/ 10^5 cells was chosen because the number of small clusters of healthy individuals did not exceed $600/10^5$ cells. In two of these groups there was a high remission rate; in group A 12/18 patients and in group B 11/16 patients achieved complete remission, while only 2/12 patients in group C achieved complete remission and 1/10 patients in group D. The median ages in groups A, B and C were somewhat above 40 years but in group D the patients were older with a median age of 59 years. The remission frequency in group (A+B) (23/34) was significantly higher than that in group (C+D) (3/22) ($p < 0.001, \chi^2$ -test). This was true also when only patients below 60 years were considered (only 1/13 patients above

60 years achieved remission) and for the 33 patients treated with TRAP. Bone marrow cells from 12 patients with ALL showed either low colony growth (group A) or no growth (group D) but there was no difference in remission rate between the two groups. Thus, the in vitro growth pattern of bone marrow cells had a prognostic value in ANLL but not in patients with ALL.

In 27 patients with ANLL a comparison between growth of marrow and blood cells showed a significant correlation in cluster formation ($r = +0.96$, $p < 0.001$). Labeling index (LI) of myeloblasts was determined in 32 patients and the mean value was $14.8 \pm 7.8\%$ (S.D.). There was a tendency for higher remission rates in patients with LI below 15 per cent but no significant predictions could be drawn, even if only the TRAP-treated patients were considered.

Addendum

The survival time of the patients treated with TRAP has been re-evaluated three years after completion of the study. The median survival times were 8.8, 18.6, 6.3 and 6.4 months for group A, B, C and D respectively. It is obvious that patients with growth pattern B had the initially best survival rate with all nine patients alive after one year while 8/13, 5/6 and 4/5 were dead in group A, C and D respectively. On the other hand, the two year survival was equal in the four growth pattern groups ($\sim 20\%$).

The survival times in relation to LI of patients treated with TRAP achieving remission has also been reevaluated. The four patients with LI above 15 per cent had a median survival of 8 months while the 10 patients with LI below 15 per cent had a median survival of 18 months.

THE RELATIONSHIP BETWEEN GROWTH IN AGAR, KARYOTYPE AND PROGNOSIS IN ACUTE LEUKAEMIA (II)

As an extension of previous work the question was raised if the cytogenetic pattern was related to the growth pattern in agar and if a combination of the two parameters improved the prognostic judgement. The agar culture technique was the same as in the previous study (I). The chromosomes were studied in direct bone marrow preparations and/or

after short term cultures without mitogen and stained by means of the trypsin-Giemsa banding technique (142). Cytogenetically the patients were divided into three categories: NN, normal metaphases only, AN, both abnormal and normal metaphases and AA, abnormal metaphases only.

Seventy-nine patients with ANLL, including 11 patients with secondary leukemia and 6 patients with an earlier preleukemic syndrome, were studied at the time of diagnosis. A clear association between cytogenetic pattern and growth pattern was seen. A higher frequency of NN-patients was found in growth pattern group (A+B) (33/55) compared to group (C+D) (4/24) ($p < 0.001$). Conversely, the incidence of AA-patients was higher in group (C+D) (12/24) than in group (A+B) (10/55) ($p < 0.005$). The frequency of AN-patients was the same in group (A+B) and (C+D). The non-random changes of chromosomes 5, 7, 8 and 21 and specific aberrations, $t(9;22)$ and $t(15;17)$ were evenly distributed between group A, B, C and D. Abnormalities of chromosomes 3 and 12 were only found in group (A+B) usually as a loss of material from the short arms of the chromosomes. The chromosome aberrations present in group (A+B) were more frequent per patient than those of patients in group (C+D), where most patients showed chromosome changes. Thus, 11/22 patients in group (A+B) had three or more aberrations per patient as compared to only 2/20 patients in group (C+D) ($p < 0.005$).

The prognosis was evaluated in 37 patients considered to have received optimal chemotherapy. The remission frequency was 27/37 (73 %) and the median age of the patients was 38 years (15-69 years). The growth pattern in agar but not the cytogenetic pattern had prognostic value in predicting remission. Thus, the remission rate in group (A+B) was 23/28 compared to 4/9 in group (C+D) ($p = 0.04$) but the survival time did not differ significantly between the two groups.

The remission frequency of NN patients (18/21) and of AN and AA patients (9/16) was not significantly different. Eight AN patients had shorter median survival - 8 months ($p=0.02$), than twentyone NN patients -14.2 months, and eight AA patients - 14 months. The combination of growth pattern and chromosome analysis did not improve the prognostic judgement.

A multivariate analysis of the influence of age showed no correlation to remission frequency, growth pattern or cytogenetic pattern in these 37 patients.

Twenty-five patients with ALL were also studied. No correlation between growth pattern and chromosomal pattern was found. The two parameters had no prognostic implications in ALL.

PREDICTION OF RESPONSE TO CHEMOTHERAPY IN ACUTE LEUKEMIA BY IN VITRO DRUG SENSITIVITY TESTING ON LEUKEMIC STEM CELLS (III)

We investigated the possibility to apply the in vitro clonogenic assay of leukemic stem cells for in vitro drug sensitivity tests and correlated the in vitro results to in vivo response to therapy.

Twenty-four patients with AML were studied; 19 at diagnosis before therapy and one patient after five courses of chemotherapy. In four patients no growth in vitro excluded them from further analysis and three patients were not treated.

Bone marrow cells were incubated with fresh solutions of ara-C and daunorubicin (DNR) for one hour at concentrations ranging from 0.15-2.4 $\mu\text{g/ml}$ ara-C and 0.018-0.30 $\mu\text{g/ml}$ DNR. After washing twice the cells were cultured in agar at 3×10^5 cells/ml and clusters (10-40 cells) and colonies (>40 cells) counted after 10 days of incubation. The number of clusters and colonies were plotted versus drug concentration on a semilogarithmic paper, and the slope of the curve was used for calculation of LD_{50} -values (15, 29). The data was also plotted in linear box diagrams and the area under the curve, AUC, which represents the fraction of surviving clonogenic cells, was calculated. AUC-values range from 0-1.0; a low value signifies drug sensitive cells and a value close to 1.0 signifies drug resistant cells. Drug concentrations were chosen to fall within the range achieved in plasma during induction of remission (3, 6, 47, 48, 204, 209).

In twelve patients the fraction of clonogenic cells in S-phase was determined with the ^3H -thymidine suicide method and compared with the data obtained with ara-C.

In seventeen patients treated with TRAP the in vitro sensitivity to ara-C and DNR could be correlated to the in vivo response to therapy. Patients who entered remission (11/17) had significantly higher sensitivity to ara-C ($p < 0.005$) and to DNR ($p < 0.02$) than patients who not entered remission (6/17). The normal AUC-values were 0.69 ± 0.08 (mean $\pm$ S.D) and 0.56 ± 0.10 for ara-C and DNR respectively. Patients who entered remission had AUC-values 0.59 ± 0.16 (ara-C) and 0.56 ± 0.17 (DNR), and patients who not entered remission had AUC-values 0.86 ± 0.12 (ara-C) and 0.78 ± 0.14 (DNR). All patients who achieved remission, with one exception, had normal or increased sensitivity to both drugs, whereas patients who not achieved remission, with one exception, had decreased sensitivity to one or both drugs. The exceptions to the general agreement between in vitro drug sensitivity and response to therapy emphasize the fact that other factors than drug sensitivity contribute to the clinical outcome.

Because ara-C kills cells during DNA-synthesis a correlation between the number of surviving clonogenic cells after exposure to ara-C and ^3H -thymidine would be expected. However, in six patients the results suggested that some clonogenic cells were metabolically resistant to ara-C and in at least one patient ara-C caused a greater reduction of clonogenic cells than ^3H -thymidine. Combinations of ara-C and DNR reduced the number of clonogenic cells only when the cells were sensitive to one or both of the drugs tested alone; if the cells were insensitive to both drugs tested alone a combination of the drugs did not result in increased reduction.

It is concluded that in vitro drug sensitivity of clonogenic cells in AML reflect the in vivo response to therapy in a majority of the patients and may possibly be developed to form the basis for an individualized chemotherapy in AML.

OSCILLATIONS OF MARROW CULTURE GROWTH IN ACUTE
MYELOID LEUKEMIA DURING REMISSION INDUCTION AND REMISSION (IV)

Marrow cells from patients with leukemia mostly show abnormal growth in agar at the time of diagnosis. Do any alterations in growth characteristics occur during the course of the disease and if so could these changes increase the understanding of how remission and relapse occur? Eight patients with AML were studied, with agar culture of marrow cells as described in paper I and II, during induction of remission, remission and relapse. Induction therapy consisted of TRAP and the maintenance therapy was TRAP alternating with COAP. Cyclic changes of the number of both clusters and colonies were observed during the induction of remission. Usually a low colony and cluster formation was observed about a week after given chemotherapy. Peaks of clonogenic cells were mostly seen just before the start of a new course of therapy. The therapy probably induced these fluctuations but similar oscillations could be noticed even when no chemotherapy was administered both during induction of remission and in remission. Usually, but not always, the numbers of clusters and colonies fluctuated in a parallel fashion.

Regardless of growth pattern at diagnosis, remission was preceded by a short phase of low or minimal growth of bone marrow cells. In one patient (CS) with an initial very high number of small clusters, the bone marrow cells ceased to grow after 3 courses of TRAP and did not regain the clonogenic capacity. The patient died 3 months later without achieving remission.

When complete remission was achieved the growth pattern showed normal features sometimes with an increased number of clonogenic cells. In some patients oscillations with longer intervals than during induction of remission were seen during remission, probably due to the fact that the maintenance therapy courses were given only once a month. Colony formation decreased or ceased at the time of morphological diagnosis of a relapse while some oscillations in the growth of clusters were still found.

RELATIONSHIP BETWEEN MYELOBLASTS AND CLUSTER-FORMING CELLS IN ACUTE
NON LYMPHOCYTIC LEUKEMIA (V)

Is there a subpopulation of cells in leukemia with self-renewal capacity and which is capable of extensive proliferation feeding cells into the blast cell compartment? With address to this question cell kinetics were analyzed in 22 patients with ANLL. Bone marrow cell suspensions incubated with a high dose of ^3H -TdR sufficient to kill cells in DNA-synthesis, were used for both agar culture to determine the fraction of clonogenic cells in S-phase (SI_{Cl} = suicide index for clonogenic cells) and for determination of LI of the myeloblast-like cells ($\text{LI}/_{\text{Mb}}$) by autoradiography.

The percentage of myeloblast-like cells in S-phase ($13.1 \pm 8.7\%$) was significantly smaller than the percentage of cluster-forming cells in DNA-synthesis ($35.5 \pm 15.3\%$). The clonogenic cells are most likely to be found within the myeloblast-like cell population but not necessarily identical with the bulk of myeloblasts. The myeloblast-like cell population may be heterogeneous. This problem was studied by comparing the total number of clonogenic cells in S-phase/ 10^5 cells (Cl_s) with the total number of myeloblast-like cells in S-phase (Mb_s) to analyze the relationship between the two cell populations. A ratio of 1.00 for Cl_s/Mb_s would mean that all myeloblast-like cells in S-phase form clones in agar. The total number of clonogenic cells in DNA-synthesis was always lower than the total number of blast-like cells in DNA-synthesis. The ratio varied between 0.53-0.0006. In two patients high ratios, 0.53 and 0.45, suggested that a majority of myeloblast-like cells in S-phase produced clusters in agar. The other patients showed decreasing plating efficiency for blast-like cells in S-phase indicating non-identity of clonogenic cells and blast-like cells or modification of the growth pattern due to environmental factors and variations in death rate during culture.

Serial studies in three patients showed fluctuations of the S-phase fraction of the clonogenic cells and of the blast-like cells. These oscillations were often non-parallel suggesting non-identity or differences in growth regulation of the two cell populations during the course of the disease.

GENERAL DISCUSSION

Despite the development of new therapeutic regimens with increasing remission frequencies, AML is still a disease with less than twenty per cent long time survivors. The basis for the therapeutic improvements has mainly been the development of new drugs and drug combinations and has so far been less dependent on the understanding of the biology of leukemia. In vitro culture techniques for clonogenic granulopoietic stem cells have held a promise to be powerful means to study the growth and differentiation of normal and leukemic progenitor cells. Such methods have so far been the methodological basis for the current models of the physiology of normal hematopoiesis. If the results of in vitro studies are relevant to the understanding of the pathogenesis of acute leukemia, they should reflect the course of the disease, response to therapy and other clinical signs. The aim of this thesis was to contribute to this understanding by investigation of the relationships between in vitro growth pattern, chromosome analysis and in vitro drug sensitivity and the response to therapy and clinical course of AML.

The growth pattern of leukemic bone marrow cells had a definite prognostic value. Colony formation or a "normal" number of small clusters without colonies signified a higher remission rate than an excessive number of clusters or no growth at all. In order to evaluate a patient population as homogeneous as possible we have excluded patients with secondary leukemia or an earlier preleukemic syndrome. The therapy was not uniform in our study, principally two different treatments were used. However, we have evaluated the prognostic implications for the uniformly treated patients separately. As pointed out previously the classification of growth pattern is very divergent in earlier studies (cf table 1 page 16). The question is raised whether the different classifications are as dissimilar as they seem to be at first sight or if the common features prevail. An attempt to compare other studies to the present study based on our classification of growth pattern is shown in table 2. We counted colonies on day 10 when their number reached a maximum, which explains the fact that we found a larger fraction of patients with colony formation than reported by investigators counting colonies on day 7 (112, 132, 150). Our proportion of patients with colony formation is in accordance with

Table 2. Comparison between the present and previous studies using the growth pattern groups of the present study.

Reference	No pts	No of therapies	Incubation days	Remission rate %	Growth pattern groups. A	B	No CR/total number C	D	% CR
Gustavsson et al. 1981	56	4	7, 10	46	12/18 (67)	11/16 (69)	2/12 (17)		1/10 (10)
	33	1 (TRAP)	7, 10	67	10/13 (77)	9/9 (100)	2/6 (33)		1/5 (20)
	33	1 (TRAP)	7	67	4/5 (80)	12/13 (92)	4/8 (50)		2/7 (29)
Knudtzon 1977 (112)	30	9	7	53	1/3 (33)	3/5 (60)	4/12 (35)		8/10 (80)
Beran et al. 1980 (16)	24	2	7	54	6/14 (43)	1/1 (100)	0/1 (0)		6/8 (75)
Kirshner et al. 1982 (110)	30	1	14	60	5/12 (42)	4/5 (80)	3/6 (50)		6/7 (86)
Moore et al. 1974 (150)	108 (83 pts treated)	6	7	36 (46 pts treated)	7/18 (39)	?	30/77 (39)	?	2/13 (15)
Robinson et al. 1975 (178)	19	2	14	26	5/10 (50)	-	-		0/9 (0)
Chervenick 1975 (39)	33	2	14	42	2/8 (25)	?	1/6 (17)	?	11/19 (58) ¹
Vincent et al. 1977 (206)	38	2	14	42	3/12 (25)	?	0/4 (0)	?	13/22 (59)
Mertelsmann et al. 1981 (132)	96	2	7	59	7/16 (44)	?	40/55 (73) ²	?	10/25 (40)

CR, complete remission

¹ including cultures with clusters of 5-10 cells and < 50 clusters/10⁵ cells.

² Some of these patients would be included in group A in the present study.

the proportion found by scoring cultures on day 14 (110, 178, 206). If we used data from day 7, only 5/13 TRAP-treated patients remained in group A (cf table 2) but the significantly different remission rates between group (A+B) (16/18) and group (C+D) (6/15) was still found ($p = 0.004$). Scoring of the plates on day 14 only has certain disadvantages; small clusters disintegrate and disappear to a large extent after day 7-8 and therefore, in some studies (39, 46, 110, 206) cultures classified as "no growth" may have contained small clusters on day 7 and would have been classified as group B according to our criteria.

The finding of colony formation as a favorable sign agrees with some reports (150, 178) but not with others (16, 39, 110, 112, 206). The group with colony formation seems to be very heterogeneous with growth of a few to an excessive number of colonies. It has been claimed that a prognostically favorable subgroup with "normal" ratio between clusters and colonies can be identified within this group probably representing residual normal granulocytic progenitor cells (150, 191). When comparing day 7 cluster:colony ratio there is a significant difference between our own patients achieving remission (mean ratio, 32.5) and patients failing to attain remission (mean ratio, 373). However, we have no data to decide if colony formation in combination with a normal cluster:colony ratio indicates persisting normal granulopoiesis. The therapeutic regimens used during the years have impact on the remission rate and influence the prognostic value of the growth pattern. In patients with colony formation it was observed that 0/5 patients treated without daunorubicin achieved remission while 7/10 patients given daunorubicin obtained remission (132). Most studies include both patients with and without treatment with daunorubicin which might be a reason for the divergent conclusions.

A consistent finding among most studies is the high remission rate in patients with growth of a low-moderate number of small clusters and the poor prognosis in patients with excessive growth of clonogenic cells. Differences in methodology may explain the lack of this correlation in two studies (46, 96).

The finding of no growth as an unfavorable sign related to a low remission rate corresponds with some (150, 178) but not with other reports (39, 110, 112, 191, 206). In three of the latter (39, 110, 206) the plates were scored on day 14 when small clusters have disappeared to a variable extent and in one study (39) the "no growth"

group included an unknown number of patients with growth of cell aggregates of 5-10 cells or less than 50 clusters/ 10^5 seeded cells similar to group B in our study. In another study (191) the group of "no growth" includes patients with a low incidence of colonies and clusters similar to group A in our study. Another report (112) also noted no growth as a favorable pattern, but as many as nine different treatment regimens were used.

Although the remission frequencies in group A and B were about the same, the one year survival for patients in group B was superior to group A as well as to group C and D. There is only one study where a relation between growth pattern and duration of remission has been documented earlier (106). The latter comprised two growth pattern groups with the same high remission rates but the group with growth of small clusters showed longer remissions. In other studies (39, 110, 112, 132, 178) no relation between growth pattern and survival time could be observed.

We found the cytogenetic pattern related to growth pattern in that sense that patients with normal metaphases (NN) mostly showed favorable growth patterns (A or B) while patients with abnormal metaphases only predominated in the unfavorable growth pattern groups (C or D). In contrast Moore et al. (150) found no correlation between euploid or aneuploid leukemias and the in vitro growth pattern but their information on the chromosome analysis is incomplete. The number and types of the chromosome aberrations in the present study are consistent with other reports in AML with about 50 per cent of the patients having abnormalities and 70 per cent of these patients showing non-random or specific aberrations. These non-random changes were evenly distributed among the growth pattern groups. Somewhat surprisingly we observed more frequent chromosome aberrations among patients in group (A+B) than in group (C+D). One explanation could be that patients in group (C+D) had a more advanced or aggressive disease where the clonal evolution had worked longer or faster and a more homogeneous and perhaps more malignant cell population had been selected. No significant prognostic differences between the three cytogenetical categories were observed in 37 optimally treated patients neither concerning remission rate nor survival time (except the observation that AN patients had the shortest survival but these patients were rather few). This finding contrasts with earlier

reports, where no consideration was taken to if therapy was given and whether it was uniform or not and sometimes patients still alive were excluded from evaluations. If all treated patients in the present study were considered, NN-patients had the longest median survival in agreement with previous reports (4, 81, 95, 118, 157, 183). Our conclusion is that a clear association exists between the cytogenetic pattern and the growth pattern but that a combination of these measurements does not improve the prognostic prediction.

A multivariate analysis in the optimally treated patients showed no correlation between age and remission frequency, cytogenetic pattern or growth pattern. Most studies have observed a low remission rate for elderly patients. Our 37 evaluated patients included few elderly patients; median age was 38 years (15-69) with only 3 patients above 60 years and 7 patients above 50 years. This age distribution may be the reason why no influence of age was observed.

In ALL none or very sparse growth of bone marrow cells occur in agar with a few colonies and/or clusters. These cell aggregates most likely represent residual normal granulopoietic progenitor cells. This assumption is strengthened by the observation of normal granulocytic maturation in vitro, a normal ratio of clusters to colonies and a normal density distribution of clonogenic cells (150). If we strictly apply the criteria of division into separate growth subclasses used in AML, the ALL patients would be distributed into group A, B and D. However, this classification is probably not relevant for ALL because of the above reasons and subsequently we found no difference in the clinical outcome between the different growth pattern groups. Furthermore, we observed no association between cytogenetic pattern and growth pattern in ALL.

Labeling index of myeloblasts seemed to have no value in predicting complete remission in agreement with most other studies (5, 45, 116, 205, 208). Patients achieving complete remission which initially had a high LI survived for a shorter time than patients with a low LI, also consistent with other reports (45, 93, 208). This observation probably reflects a high growth rate of the leukemic cell population and may imply that these patients have a high risk of an early relapse.

Compared to other studies we found relatively high labeling indices, which may depend on our use of low density cells (< 1.077 g/ml) instead of unseparated bone marrow cells.

We applied the clonogenic assay for testing in vitro drug sensitivity of leukemic stem cells and found a correlation between the drug sensitivity of the leukemic cells and the clinical response to therapy consistent with previous reports (163, 171). In one study (171) the leukemic cells were incubated one hour with two different concentrations of either ara-C or DNR or a combination of both drugs prior to culture in agar. With only two drug concentrations no survival curves can be constructed nor calculation of LD_{50} or AUC. Only patients sensitive to both drugs entered remission. In contrast we found one patient whose cells were extremely sensitive to ara-C but almost resistant to DNR but the patient achieved remission. In the other study (163) leukemic cells were exposed to a single dose of different drugs and the results compared with simultaneously studied normal controls and expressed as a sensitivity index, SI, (the surviving fraction of normal CFU_c : the surviving fraction of leukemic CFU_c). Patients having SI greater than 1.0 entered remission, whereas patients with SI less than 1.0 did not. However, calculation of a sensitivity index can be questioned since the authors showed a great variation in drug sensitivity of normal CFU_c and the outcome of their tests will depend on both the variable sensitivity of the normal control and the sensitivity of the leukemic cells.

In another report (128) the cytostatic drug effects on blast progenitor cells in peripheral blood stimulated by PHA-LMC were evaluated. A wide patient to patient variation in sensitivity to ara-C and doxorubicin was seen, although the sensitivity in the same patient seemed to be stable or only slowly changing over a period of 16 months (127). The sensitivity to ara-C predicted achievement of remission while the sensitivity to doxorubicin did not.

The most important finding is the correlation between in vitro drug sensitivity and response to therapy, which shows that the in vitro tests reflect important properties of the in vivo interactions between chemotherapeutic drugs and the leukemic cells. However, some limitations must be considered; nutritional factors may modify the cytotoxicity (144) as well as the levels of the enzymes that activate

or inactivate the drug. For instance, as also observed by others (171), we noticed a discrepancy between the reduction of clonogenic cells caused by tritiated thymidine and ara-C, which suggested that in some cases the clonogenic cells are metabolically resistant to ara-C despite the fact that they are in DNA-synthesis. Furthermore, there may not be a direct relationship between the plasma level and the intracellular concentration of the drug as has been shown for daunorubicin (164). One study has shown that both normal and leukemic cells are less sensitive in vitro to doxorubicin than to daunorubicin (29) which does not agree with clinical experience. In addition, patients are treated with a combination of drugs in a certain sequence which is difficult to achieve in vitro. Nevertheless, in vitro drug sensitivity tests may be important for selection of the drugs effective in combination therapy, avoiding ineffective but toxic drugs and for screening of new chemotherapeutic agents. In vitro tests may also identify patients with a poor chance of response to current therapy justifying attempts at alternative treatments.

The oscillations of cluster and colony formation which we found during the course of the disease are probably due to recruitment of resting leukemic and normal cells into active proliferative phase upon chemotherapeutic perturbation. We found that shortly after given chemotherapy the number of clonogenic cells was reduced while the fraction of cells in S-phase was high. Immediately prior to the following course of therapy several observations revealed an increasing number of clonogenic cells and a decreasing S-phase. Similar results have been reported by others (86).

Interactions between leukemic and normal cells may be of considerable significance in the pathophysiology of leukemia. Substances inhibiting the growth of normal granulopoietic cells but not the growth of leukemic cells have been identified (23, 26, 160). These inhibitors may provide a growth advantage for the leukemic cells and contribute to the expansion of the leukemic cell mass at the expense of normal cells. Chemotherapy reduces the number of blast cells and may subsequently diminish their inhibitory effects which allow normal granulopoietic precursors to proliferate as evidenced by the return of normal colony growth. The oscillations during induction of remission may represent a competition between leukemic and normal cells and might be regarded as potential remissions. Remission in most cases

occurred after a short period of minimal or no growth in vitro. This finding is in accordance with the clinical observation that bone marrow hypoplasia precedes remission in a majority of the patients. On the other hand, we have observed a few patients where colony and cluster growth did not return after treatment and these patients died in aplasia. The transient leukemic growth pattern observed in some cases during remission may suggest the persistence of otherwise undetectable leukemic cells in the marrow during prolonged remission. The oscillations during remission may also reflect a competition between leukemic and normal cells where the outcome depends on which cell population will have the proliferative advantage. At such occasions chemotherapy could depress the leukemic cells and facilitate the reestablishment of normal hematopoiesis and possibly prevent relapse.

The proliferative rate of AML cells at diagnosis is considered to be low compared with normal blast cells judging from studies on ^3H -TdR labeling (40, 75, 108). However, a subpopulation among the morphologically recognizable blast cells may have a high proliferative capacity and maintain the leukemic cells. Most leukemic blasts may have lost the ability to divide and become end cells. If so, the rapidly growing cells would be diluted by all the non growing cells resulting in a low LI. In support of this view is the observation of blast cells of different sizes where large blasts show a normal LI and small blasts a very low LI (75). By culturing cells from patients with leukemia in agar we found a rapidly proliferating cell population of probable leukemic origin whose S-phase fraction was three times greater than that of the mass of blast-like cells in agreement with other reports (170). When comparing the total numbers of proliferating clonogenic cells and myeloblasts respectively our data suggested a similarity between blast-like and clonogenic cells in two cases. In the twenty remaining patients the clonogenic cells represented a small minority of the blast-like cells. Since the plating efficiency is unknown the size of the clonogenic subpopulation cannot be determined exactly. The cell population growing in agar is probably more important in terms of response to therapy than the bulk of blast cells. Therefore it seems plausible that LI for myeloblasts do not correlate with the therapeutic response. It would have been of interest to estimate if SI of clonogenic cells had any prognostic value but too few data were available to make a comparison possible.

SUMMARY

The prognostic value of growth pattern in vitro, cytogenetic pattern and in vitro sensitivity to chemotherapeutic agents of bone marrow cells in AML was studied. In addition, variations in the growth pattern during the course of the disease and the relationship between clonogenic cells and blast-like cells were studied.

1. The growth pattern in agar of 56 patients with AML could be divided into four groups. Patients with growth of colonies (A) or a normal number of clusters without colonies (B) had significantly higher remission rates (23/34) than patients with an excessive number of clusters (C) or no growth in vitro (D), (3/22) ($p < 0.001$). Patients in group B had initially the best survival rate but the two year survival was equal in the four growth pattern groups. In ALL the growth pattern had no prognostic significance.

2. An association between cytogenetic pattern and growth pattern was found in 79 patients with AML. A higher frequency of NN-patients (normal metaphases only) was found in growth pattern group (A+B) (33/55) compared to group (C+D) (4/24), ($p < 0.001$). The incidence of AA-patients (abnormal metaphases only) was higher in group (C+D) (12/24) than in group (A+B) (10/55), ($p < 0.005$). Aberrations of chromosomes 3 and 12 were found only in groups A and B. Chromosome abnormalities were more frequent per patient in groups A and B than in groups C and D. A combination of growth pattern and cytogenetic pattern did not improve the prognostic judgement.

3. The in vitro sensitivity of clonogenic cells to ara-C and DNR was studied in 20 patients with AML. Patients achieving remission had significantly higher sensitivity to both ara-C and DNR than patients not entering remission ($p < 0.005$ and $p < 0.02$ respectively).

4. Serial studies of in vitro growth during the course of AML showed marked oscillations in the number of clusters during induction of remission. The oscillations are probably due to recruitment of resting leukemic and normal cells into active proliferation upon chemotherapeutic perturbation and may reflect a competition between leukemic

and normal cells. Remission was in most cases preceded by a phase of no growth in vitro and during remission growth showed normal features. Colony formation decreased or ceased at the time of relapse.

5. The number of clonogenic cells and blast-like cells in DNA-synthesis was analyzed in 22 patients. The percentage of blast-like cells in S-phase ($13.1 \pm 8.7 \%$) was significantly smaller than that of cluster-forming cells ($35.5 \pm 15.3 \%$). In two patients a comparison of the total number of cluster-forming cells and blast-like cells in S-phase suggested identity of the two cell populations. In the remaining patients the same comparison showed that cluster-forming cells represent a small subpopulation of the blast-like cells.

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The Prognostic Significance of in Vitro Bone Marrow Growth Pattern in Acute Non-Lymphocytic Leukaemia

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The growth pattern of bone marrow cells in agar culture was studied in 56 patients with untreated acute non-lymphocytic leukaemia. 4 different growth patterns were recognized: (A) colony and cluster formation, (B) growth of small clusters (3–20 cells) only but less than $600/10^5$ cells, (C) growth of small clusters only but more than $600/10^5$ cells, and (D) no growth in vitro. Patients in group A and B had a significantly higher remission rate (23/34) than patients in group C and D (3/22). This was true also when only patients below 60 years of age were considered. The median survival time of patients in group A and B was 7.3 months and 9.4 months, and in group C and D 4.0 months and 3.3 months. Identical growth patterns were found for marrow and blood cells in 21 of 27 patients studied and the number of clusters in blood and marrow showed a significant correlation ($r = +0.96$, $P < 0.001$). The mean ^3H -labelling index (LI) of myeloblasts determined in 32 patients was $14.8 \pm 7.8\%$ (SD). Patients with LI below 15 % tended to have a higher remission rate than patients with LI above 15 %. It is concluded that the in vitro growth pattern at diagnosis of bone marrow cells from patients with ANLL is of value in detecting patients with a poor response to current therapeutic protocols. Such information could possibly lead to alternative cytostatic regimes in these patients.

Key words: acute leukaemia – agar culture – prognostic factors

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The proliferative capacity of granulopoietic progenitor cells can be studied in vitro by the semi-solid agar culture technique (Ichikawa et al 1966, Bradley & Metcalf 1966). In the presence of an appropriate stimulus, colony stimulating activity (CSA), these cells (CFU-c) proliferate and produce colonies of granulocytes and monocytes (Chervenick & Boggs 1971, Craddock et al 1973). The in vitro growth pattern of CFU-c in

acute leukaemia is abnormal and variable; colony formation (cell aggregates > 40 cells) is usually absent but various amounts of clusters (3–20 cells) develop or there is no growth at all (Greenberg et al 1971, Moore et al 1973, 1974). The abnormal boyant density, failure to differentiate in vitro and karyotypic anomalies (Duttera et al 1972, Moore & Metcalf 1973, Moore et al 1973, 1974) have been taken as evidence

for the leukaemic origin of colonies and clusters in acute leukaemia.

Several studies have tried to correlate the in vitro growth pattern to prognostic outcome (Moore et al 1974, Chervenick 1975, Curtis et al 1975, Spitzer et al 1976, Hörnsten et al 1977, Knudtzon 1977, Vincent et al 1977, Beran et al 1980). The results have been variable depending on differences in culture technique, sources of CSA, classification of growth pattern and therapy, although several common features can be identified. For instance, the growth of colonies or small clusters is usually related to a favourable prognosis whereas no growth in vitro or an excessive production of small clusters relate to a poor prognosis. There are, however, several exceptions to this rule.

In the present study which included 56 patients with acute non-lymphocytic leukaemia (ANLL) 4 different growth patterns could be distinguished. There were significant differences in the remission rate between the 4 groups. It is concluded that the in vitro growth pattern in combination with other prognostic factors is a useful prognostic tool in acute leukaemia.

MATERIALS AND METHODS

Patient selection and treatment

56 consecutive patients with untreated non-lymphocytic leukaemia were studied between Jan 1975 and June 1980. The patients had no known underlying haematologic abnormality. The diagnoses were according to the criteria of Galton & Dacie (1975) (Table 1). The age ranged from 15 to 84 years (median 44). There were 29 females with a median age of 50 years and 27 males with a median age of 43 years. All patients received chemotherapy and only patients surviving for more than 7 d were included in the study. Remission induction regimes varied during the 5-year period of the investigation. 33 patients were treated with cytosine

arabinoside i.v., thioguanin per os and prednisone per os during 5 d, with daunorubicin i.v. on the first day (TRAP). The treatment was repeated if possible every tenth day. 2 patients were treated with cytosine arabinoside i.v. during 5 days and daunorubicin i.v. on the first day. 1 patient was given cyclophosphamide i.v., cytosine arabinoside i.v. and prednisone per os during 5 d with vincristine i.v. on the first day (COAP). 20 patients were treated with prednimustine per os daily with or without vincristine i.v. every 10th day. Patients induced with TRAP-treatment continued with TRAP twice alternated with COAP twice, with 1 cycle each month. Patients achieving remission on prednimustine treatment continued with prednimustine in a lower dose.

For comparison bone marrow cells from 12 patients with acute lymphocytic leukaemia (ALL) were studied during the same period (Table 2). This group included 3 females and 9 males with a median age of 31 years. The induction treatment varied as indicated in Table 2.

Complete remission was defined as normal bone marrow cellularity with less than 5% blast cells, return of neutrophil counts to $1.5 \times 10^9/l$ or greater and platelet counts to at least $100 \times 10^9/l$.

Collection of bone marrow and blood

2–3 ml bone marrow was aspirated from the sternum and collected in 3 ml of McCoy's medium with 100 U of heparin and 75 U of Varidase (Lederle Laboratories, Pearl River, NY). Marrow particles were disrupted by repeated suction through a needle (0.8 × 80 mm). 10–20 ml heparinized blood was collected.

Cell separation

7 ml of marrow cells suspended in McCoy's medium were layered on top of 5 ml Isopaque-Ficoll solution with a density of 1.077 g/ml (Bøyum 1968) in 16 ml plastic tubes. The tubes were centrifuged in a swing out rotor for 10 min at 70 g and for 15 min at 700 g. The cells lighter than 1.077 g/ml collected at the interphase were washed 3 times in McCoy's medium and used for culture in agar. 1 part blood was mixed with 1 part 2% Dextran 250 (Pharmacia, Uppsala, Sweden) in 0.15 M NaCl and was allowed to stand for 30–45 min at room temp. 30 ml of Dextran plasma were layered on top of 20 ml Isopaque-Ficoll solution in 50 ml sterile plastic tubes and then centrifuged and washed as described above.

Marrow cells from some patients were also separated on an isoosmolar Isopaque-Ficoll solution ac-

TABLE 1
Data on 56 patients with acute non-lymphocytic leukaemia

Patient (sex/age)	Diagnosis	Treatment	Remission	Survival (days)	Growth pattern	ClFU-c S-ph.(%)	Hb L.I (%)
LO (F/19)	M 5	TRAP	CR	207*	A	26	-
AH (F/69)	M 4	COAP	CR	244	A	-	34
LOF (M/20)	M 0	TRAP	CR	461	A	-	-
TF (M/26)	M 4	TRAP	CR	296	A	12	14
HN (M/30)	M 5	TRAP	CR	216	A	-	27
JN (M/36)	M 2	PM+VCR	CR	299	A	-	-
UK (M/43)	M 2	TRAP	CR	223*	A	-	-
SS (M/46)	M 6	TRAP	CR	686	A	38	3
KK (M/49)	M 4	TRAP	CR	171	A	39	13
KA (M/52)	M 4	TRAP	CR	1063	A	-	-
GS (M/52)	M 5	TRAP	CR	174*	A	-	-
ER (M/59)	M 4	TRAP	CR	206	A	54	11
SL (F/38)	M 0	TRAP	NR	260	A	-	-
HS (F/63)	M 2	TRAP	NR	56	A	12	29
TA (F/64)	M 5	PM+VCR	NR	76	A	-	-
MK (F/74)	M 4	PM	NR	24	A	-	20
AA (M/15)	M 5	PM+VCR	NR	52	A	-	-
KAA (M/24)	M 2	TRAP	NR	222	A	-	-
GS (F/21)	M 4	TRAP	CR	528	B	-	16
ACW (F/26)	M 6	TRAP	CR	404	B	26	14
VA (F/34)	M 2	TRAP	CR	263*	B	-	-
RBK (F/39)	M 1	Ara-C+DNR	CR	251	B	-	10
IJ (F/40)	M 2	TRAP	CR	499	B	23	5
DZ (F/44)	M 1	TRAP	CR	1424*	B	-	8
RS (M/38)	M 2	TRAP	CR	606	B	37	7
GH (M/38)	M 4	TRAP	CR	251*	B	38	-
AS (M/39)	M 1	PM+VCR	CR	298	B	-	-
AO (M/41)	M 1	TRAP	CR	587	B	37	4
BP (M/44)	M 1	TRAP	CR	384	B	-	-
AP (F/51)	M 4	PM+VCR	NR	168	B	-	-
CJ (F/64)	M 4	PM	NR	32	B	-	26
NEA (M/48)	M 5	PM+VCR	NR	158	B	-	16
JB (M/55)	M 4	PM+VCR	NR	130	B	-	-
SA (M/64)	M 3	PM	NR	68	B	-	-

TABLE 1 (continued)

Patient (sex/age)	Diagnosis	Treatment	Remission	Survival (days)	Growth pattern	ClFU-c S-ph.(%)	Mb LI (%)
IK (F/23)	M 4	TRAP	CR	270	C	-	19
LS (M/44)	M 4	TRAP	CR	1187	C	-	-
CP (F/15)	M 4	TRAP	NR	8	C	-	19
MK (F/27)	M 4	Ara-C+DNR	NR	14	C	-	19
CS (F/41)	M 2	TRAP	NR	121	C	45	10
IJ (F/50)	M 2	PM+VCR	NR	118	C	22	4
RS (F/53)	M 2	PM+VCR	NR	43	C	23	12
AS (F/59)	M 2	TRAP	NR	187	C	-	-
TK (F/65)	M 4	PM	NR	88	C	-	-
AS (F/68)	M 4	PM	NR	43	C	8	25
KHB (M/32)	M 2	PM+VCR	NR	128	C	-	-
Jö (M/39)	M 4	TRAP	NR	193	C	-	-
AA (F/44)	M 2	TRAP	CR	1116*	D	-	10
GK (F/34)	M 3	PM+VCR	NR	16	D	-	4
HL (F/51)	M 1	TRAP	NR	182	D	-	23
IJ (F/54)	M 1	TRAP	NR	154	D	-	-
MW (F/63)	M 4	PM+VCR	NR	17	D	-	21
AB (F/84)	M 2	PM	NR	43	D	-	15
AB (M/33)	M 0	TRAP	NR	280	D	-	-
HI (M/63)	M 1	TRAP	NR	192	D	-	13
KK (M/64)	M 5	PM+VCR	NR	16	D	-	13
EP (M/84)	M 2	PM	NR	30	D	-	10

The morphological diagnosis (M 0-M 6) were according to Galton & Dacie (1975). Treatments with COAP and TRAP are detailed in Material and Methods. PM, prednimustine; VCR, vincristine; Ara-C, cytosine arabinoside; DNR, daunorubicin; CR, complete remission; NR, no remission. *, patients still alive. Growth pattern: group A, colonies and clusters; group B, no colonies and < 600 small clusters/10⁵ cells; group C, no colonies and > 600 small clusters/10⁵ cells; group D, no growth. ClFU-c S-ph, cluster forming cells in S-phase. Mb LI, thymidine labelling index of myeloblasts.

cording to Wells et al (1977). After dextran sedimentation the cells were suspended in a 'light' Isopaque-Ficoll solution (density 1.046 g/ml, osmolality 285 mOs/kg H₂O and pH 7.2) (Wells et al 1977). 2 ml of cell suspension, 10⁷ cells/ml, were layered on top of 5

ml of a mixture of 6.44 ml 'light' solution plus 3.56 ml 'heavy' solution (density 1.062 g/ml, osmolality 285 mOs/kg H₂O and pH 7.2) (Wells et al 1977) and centrifuged in a swing out rotor for 30 min at 500 g. Cells with a density less than 1.062 g/ml were de-

TABLE 2
Data on 12 patients with acute lymphatic leukaemia

Patient (sex/age)	Treatment	Remission	Survival (days)	Growth pattern
SB (M/21)	PM + VCR	CR	305	A
PS (M/21)	A+VCR+P → CHOP	CR	323	A
HB (M/23)	PM + VCR	CR	68	A
CH (M/35)	COAP	CR	202	A
ILA (F/60)	PM + VCR	CR	93	A
MP (M/17)	A+VCR+P → TRAP	NR	291	A
LL (M/62)	TRAP	NR	162	A
IB (F/23)	TRAP	CR	447*	D
LP (F/26)	VCR+P → Ara-C+A	CR	701*	D
UP (M/43)	TRAP	CR	470	D
HC (M/60)	COAP	CR	617	D
AH (M/62)	PM + VCR	CR	50	D

COAP and TRAP are detailed in Material and Methods. CHOP, cyclophosphamide i.v., adriamycin i.v. and vincristine i.v. on the first day with prednisone per os during 5 days; A, adriamycin; Ara-C, cytosine arabinoside; VCR, vincristine; PM, prednimustine; P, prednisone. CR, complete remission; NR, no remission. * indicates patient still alive. Growth pattern: group A, colonies and clusters; group D, no growth.

signated A cells and cells with a density above 1.062 g/ml B cells. A and B cells were washed twice in McCoy's medium containing 5% fetal calf serum (FCS) and utilized in agar culture.

Cell culture technique

The bone marrow culture technique of Pike & Robinson (1970) was utilized with the following modifications. The feeder layers contained blood mononuclear cells, isolated on Isopaque-Ficoll 1.077 g/ml, obtained from 1 of 2 normal donors throughout the study. The feeder capacity of these 2 donors did not differ significantly. 3×10^5 cells in 1 ml of 0.5% agar (Difco

Bacto Agar) in McCoy's medium with 15% FCS were plated in 35 mm Falcon plastic Petri dishes. The upper layers contained 10^5 marrow cells in 1 ml of 0.3% agar and McCoy's medium with 15% FCS. After gelling at room temperature the plates were incubated at 37°C in a fully humidified atmosphere with 7.5% CO₂ in air.

Cells separated at 1.062 g/ml were cultured with 10^5 A or B cells in the upper layers and also in mixtures of 10^5 A plus 10^5 B cells. Alternatively, A and B cells were mixed in the same proportions as they were present in unseparated marrow and cultured at a final concentration of 10^5 cells/ml.

Cultures were scored with an inverted microscope after 7 d for small clusters of 3–20 cells, large clusters of 21–40 cells and colonies of more than 40 cells. Colonies and large clusters were counted after 10 d. Small clusters reached their maximum at 7 d and degenerated thereafter whereas colonies reached their maximum at 10 d of culture. Therefore, data presented for small clusters are from d 7 while data for colonies are from d 10 of culture. Each determination is the mean value of 3 dishes.

The range of colony incidence for marrow specimens of 9 healthy individuals was 16–65/10⁵ seeded cells and the median number was 43. For small clusters the range was 67–592/10⁵ seeded cells with a median number of 327.

In several of the patients with leukaemia (including all types of growth pattern in agar) cells were plated at increasing cell concentrations (0.5 × 10⁵, 1 × 10⁵ and 2 × 10⁵ cells/dish) and the number of cell clones produced showed a linear relation to the amount of cells plated and no plateau of the growth was observed within this range. It is concluded that the culture system provides optimal growth conditions at a cell concentration of 10⁵ cells/dish.

Tritiated thymidine suicide and labelling index

To determine the proportion of progenitor cells in DNA-synthesis the ³H-thymidine suicide technique was used. 1½ ml of cells lighter than 1.077 g/ml in McCoy's medium, 3–5 × 10⁶ cells/ml, were incubated with 75 µCi of ³H-thymidine with a specific activity of 19–21 Ci/mmol (Radiochemical Centre, Amersham, England). A control was run simultaneously without ³H-thymidine. After 30 min of incubation at 37°C the cells were washed once in 15 ml of McCoy's medium containing 100 µg/ml cold thymidine and once in medium without thymidine and then used for agar culture as described. The reduction of cluster/colony formation upon suiciding is taken as a measure of the fraction of cluster/colony forming cells in DNA-synthesis. The cell suspensions incubated with ³H-thymidine were also used for determination of the ³H-thymidine labelling index (LI) of myeloblasts by autoradiography using emulsion K2 (Ilford Ltd., Ilford, England). The smears were exposed for 1 week at 4°C and stained with May-Grünwald-Giemsa. Cells with 5 grains or more were considered as being labelled; 400 cells were counted.

Classification of growth pattern

Principally 4 different growth patterns could be dis-

tinguished in leukaemia and the patients were divided accordingly into 4 groups to correlate in vitro growth pattern to the remission rate: Group A: growth of colonies and clusters. Group B: no growth of colonies and less than 600 small clusters per 10⁵ cells. Group C: no colonies and more than 600 small clusters per 10⁵ cells. Group D: no growth in vitro of either colonies or clusters (i.e. less than 10 clusters per plate; this background cluster growth was due to cluster formation in the feeder layer). The distinction limit of 600 small clusters was chosen on the basis of the normal growth pattern where the upper limit for small clusters was approximately 600 per 10⁵ cells (see above).

Statistical analysis

Non-parametric statistical tests were used to analyze the data. When the 2 independent samples are small in size (N < 20) or when N is between 20 and 40 and one expected frequency is below 5 Fisher's exact probability test was used. When N > 40 the χ^2 test for 2 independent samples corrected for continuity and 1 degree of freedom was utilized.

RESULTS

Clinical findings

Table 1 summarizes the clinical and in vitro findings of the 56 ANLL patients. 26 of the 56 patients achieved complete remission (46%). The median survival was 28 weeks for all patients and 43 weeks for those attaining complete remission. If only patients below 60 years of age are examined 25/43 (58%) patients were found to achieve complete remission. Patients about 60 years had a poorer prognosis with only 1/13 achieving complete remission (P < 0.01, Fisher's exact probability test). There seemed to be a tendency towards a sex difference in the remission frequency; in females the remission frequency was 10/29 (34%) and in males 16/27 (59%). The same tendency was seen even if only patients below 60 years of age were considered, with complete remission in 9/20

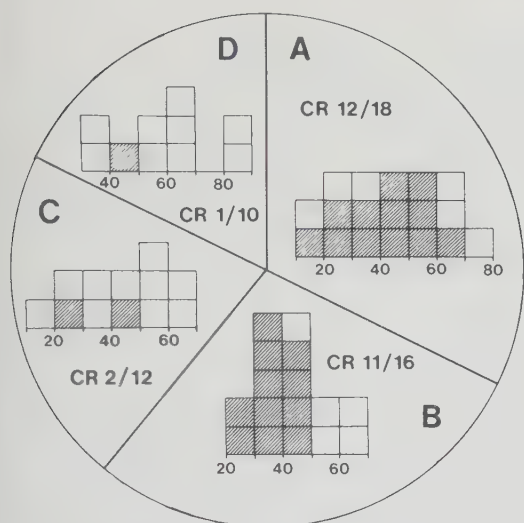


Figure 1. The distribution of 56 patients with ANLL among the four in vitro growth patterns and age distribution within each group. Each quadrant represents one patient. Hatched areas denote patients achieving complete remission.

Group A: growth of colonies and clusters.

Group B: no colonies, < 600 small clusters/ 10^5 cells.

Group C: no colonies, > 600 small clusters/ 10^5 cells.

Group D: no in vitro growth.

CR, complete remission.

females (45%) and 16/23 males (70%). These differences, however, are not statistically significant.

Relation between in vitro growth pattern and remission rate

In Table 1 the patients are listed according to growth pattern and whether they achieved complete remission (CR) or not (NR). Figure 1 illustrates the relation between in vitro growth pattern, remission rate and age distribution. Group A comprised 18 patients with 12 CR and a median age of 45 years. Group B included 16 patients with 11 CR and a median age of 41 years. In group C there were 12 patients with only 2 CR and a median age of 43

years. Group D consisted of 10 patients with 1 CR and a median age of 59 years. The remission frequency in group A and B together (23/34) was significantly higher than in group C and D together (3/22) ($P < 0.001$, χ^2 test). In Table 3 a comparison has been made between all patients and those patients under and above 60 years of age. If only patients under 60 years are considered, group A and B still have a higher remission frequency than group C and D ($P < 0.001$, Fisher's exact probability tests). The morphological diagnosis of ANLL did not show any correlation to the prognosis, nor did any morphological subgroup show a predilection for any of the growth pattern groups.

The 12 patients with ALL studied for comparison were all either group A or group D (Table 2). Only 5 of the 7 patients in group A achieved complete remission whereas all 5 patients in group D did so.

Bone marrow aspirates are to a variable degree diluted with peripheral blood and it could, therefore, be expected that high WBC would dilute the bone marrow clonogenic cells and result in poor growth. This possibility was excluded, however, since there was no relation between WBC counts and growth pattern. Patients in the non-growing group D (both ANLL and ALL) did not have higher WBC than the other patients. Furthermore, there was an excellent correlation between the number of clusters in marrow and blood (see below).

Relation between in vitro growth pattern and survival time

Figure 2 shows a life table comparing the survival time of all patients belonging to the different growth pattern groups. The median survival time of patients in group A and B was 7.3 months (3 patients are still alive) and 9.4 months (3 patients are still

TABLE 3
Comparison of remission frequency in patients under and above 60 years of age
Patients are divided into the different growth pattern groups

Growth pattern	All patients (CR/no of pat.)	Patients < 60 yrs (CR/no of pat.)	Patients > 60 yrs (CR/no of pat.)
Group A	12/18	11/14	1/4
Group B	11/16	11/14	0/2
Group C	2/12	2/10	0/2
Group D	1/10	1/5	0/5
Total	26/56	25/43	1/13

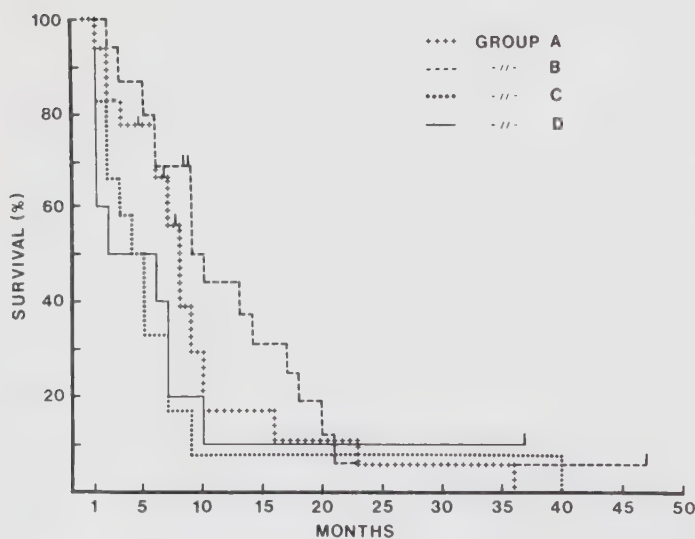


Figure 2. The survival time in months of patients belonging to the different growth pattern groups: (A, B, C, D). Vertical bars represent patients still alive. Median survival time in group A: 7.3 months, group B: 9.4 months, group C: 4.0 months and group D: 3.3 months.

alive), respectively. In group C and D the median survival time was 4.0 months and 3.3 months (1 patient is still alive), respectively. Each growth pattern group included 1 patient who survived for at least 3 years.

When the patients were divided into TRAP or prednimustine treated categories

the following median survival times were found (the number of patients are shown within brackets): TRAP group A 7.4 months (13), group B 16.6 months (9), group C 6.3 months (6), group D 6.4 months (5) and prednimustine group A 2.1 months (4), group B 4.8 months (6), group

TABLE 4
Cluster formation of separated and recombined bone marrow cells in ANLL

Patient (sex/age)	Clusters ≤1.062 g/ml (% of total)	Number of clusters per			
		10 ⁵ A cells	10 ⁵ B cells	10 ⁵ A + 10 ⁵ B (actual/expected number)	A + B (=10 ⁵)
BP (M/44)					
7706	2.3	30	700	1.58	
7803	50	1730	1015	.71	
7803	92	1770	105	1.50	1.84
7805	14	645	1010	1.27	1.38
TA (F/64)	84	1825	300	.92	
KK (M/49)	99	1455	20	1.16	
AS (F/68)	30	5090	1655	.83	1.03
RS (F/53)	74	3200	1100		.95
SW (F/74)	17	950	505		1.17
BW (M/49)	70	35	10		2.50
NEA(M/48)	94	405	230	1.07	.90
EN (F/68)					
3-20 cells	95	1815	48		.73
21-40 -"	91	880	47		.99
40 -"	100	220	0		.55
SP (F/74)	90	3	3		.66
Mean ± S.D.					
	-	-	-	1.13 ± 0.3	1.15 ± 0.5

Bone marrow cells were separated in isoosmolar Isopaque-Ficoll at 1.062 g/ml and cultured in the concentrations given in the table. Cells lighter than 1.062 g/ml were called A cells and cells denser than 1.062 g/ml were called B cells. The normal mean ± SD for clonogenic cells < 1.062 g/ml was 75 ± 6 (n = 4).

C 2.9 months (5), group D 0.6 months (5). The median age of TRAP treated patients was 41 years and 59 years for prednimustine treated patients.

Cell separation and recombination studies
 Clonogenic cells in acute leukaemia usually have a lighter boyant density than normal when separated in density gradients of

bovine serum albumin (Moore et al 1973, 1974). Density cut separation in isoosmolar Isopaque-Ficoll at a density of 1.062 g/ml was performed in 10 patients with ANLL (Table 4). 5 of the patients showed an abnormal light density shift with 90 % or more of the clonogenic cells lighter than 1.062 g/ml (normal value 75 ± 6 , $n = 4$). 3 patients were within the normal range whereas 3 patients showed a majority of cluster forming cells denser than 1.062 g/ml. In 1 patient (BP) the proportion of cluster forming cells lighter than 1.062 g/ml drastically changed from 2.3 % at diagnosis to 50 and 92 % in early relapse and back to 14 % 3 weeks prior to death.

When recombinations of light and dense cells were cultured in agar in the same proportions as they were present in unseparated samples the number of clusters was close to the expected number with a few exceptions (Table 4). Also recombination of equal amounts of light and dense cells resulted in the expected or higher than expected number of clusters.

One patient (S.P.) in group D was included in these cell separation studies but none of the separated cell fractions showed an improvement in growth capacity.

These studies taken together show that it is unlikely that inhibitory actions within the cell populations used in the standard assay would distort the results and displace any of the patients to the non-growing category (group D).

Comparison between bone marrow and blood cultures

In 27/56 patients with ANLL blood cells were cultured in agar simultaneously with bone marrow cells. 21 patients exhibited identical growth pattern for marrow and blood cells while 6 patients showed non-

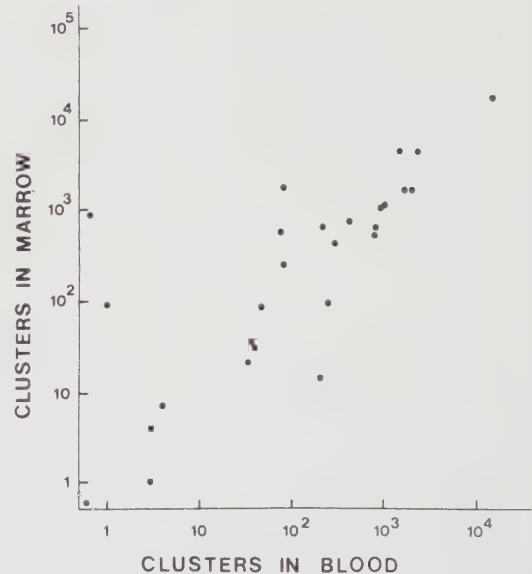


Figure 3. Relationship between clusters (small and large) in bone marrow and blood at the time of diagnosis in 27 patients with ANLL. Correlation coefficient $r = 0.96$, $P < 0.001$.

identical growth pattern. 5 of these patients grew fewer cell aggregates in blood cultures than in the marrow cultures and 1 patient showed the opposite relationship.

The correlation between the growth of clusters in bone marrow and blood was highly significant ($r = +0.96$, $P < 0.001$) (Figure 3). Any correlation for colonies could not be calculated because too few patients with growth of colonies were studied.

DNA-synthesis of myeloblasts and clonogenic cells

The labelling index (LI) of myeloblasts was determined in 32 patients (Table 1). The mean LI was $14.8\% \pm 7.8$ (SD). 11/18 patients with LI below 15 achieved complete remission. When LI was above 15 only 4/14 patients attained complete remission. The survival time of the patients with

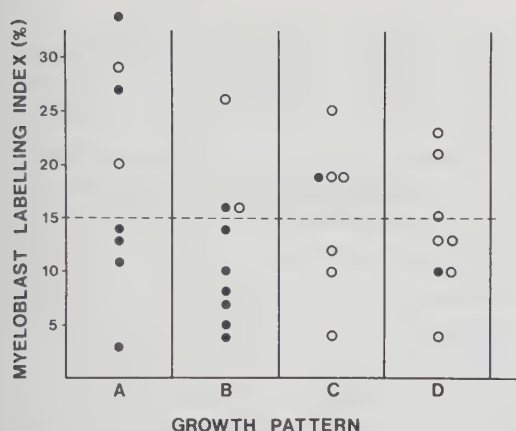


Figure 4. Myeloblast labelling index (LI) in 32 patients with ANLL at the time of diagnosis. Patients are divided into the 4 different growth patterns (A, B, C, D). Open circles show patients not achieving remission and closed circles patients achieving complete remission.

LI below 15% and achieving complete remission was not significantly longer (median 57 weeks) than in patients with LI above 15% (median 37 weeks). If the data on LI, however, were combined with the in vitro growth pattern there was a correlation with remission rate (Figure 4). Patients with growth pattern A and B with a LI below 15 showed a remission frequency of 100% (10/10) whereas 3/7 with a LI above 15 achieved complete remission ($P < 0.025$, Fischer's exact probability test).

The fraction of cluster forming cells (CIFU-c) in S-phase was determined in 15 patients (Table 1) and ranged from 8–54% with a mean of 29.3 ± 12.7 (SD). No valid prognostic significance could be ascribed to the fraction of CIFU-c in S-phase of this limited number of patients.

DISCUSSION

In this study we show that the in vitro growth pattern in agar of bone marrow cells

from patients with acute leukaemia is abnormal and can be classified into 4 groups that show different remission frequencies and survival times. Growth in agar of both colonies and clusters (group A) or growth only of a normal number of small clusters (group B) was prognostically favourable with a higher remission frequency and longer survival time compared with growth of an excessive number of small clusters (group C) or no growth in vitro (group D). Usually age is considered to be a prognostic factor in achieving complete remission in leukaemia (Gehan et al 1976), even if the reason for this is perhaps that older patients are not treated as extensively as younger patients (Brandman et al 1979). Although it is evident that the patients of the present study older than 60 years had a poorer prognosis than patients younger than 60 years this fact did not alter the prognostic value of the in vitro growth pattern; if only patients below 60 years of age are considered (Table 3) the prognostic conclusions from the growth pattern are still valid.

Our results are to a certain extent in accordance with those of Moore et al (1974) (108 AML patients, of which 83 were treated) who established a fairly good prognosis with growth of colonies and clusters or growth of small clusters only and a poor prognosis in cases of no growth in vitro. We, however, divided patients whose marrow cells grow with small clusters into 2 groups. The reason was that the cut off point at 600 small clusters/ 10^5 cells was the upper limit for small clusters in normals and that we found quite different remission frequencies in ANLL below and above this limit. An attempt to compare the present results with those of Spitzer et al (1976) (76 patients) showed some agreement in that patients with a small cluster growth have a good

prognosis. Both groups A and D of our study would fall into the group without leukaemic proliferation in the study of Spitzer et al (1976) having a favourable prognosis. These authors, however, included ALL and acute undifferentiated leukaemia in their material (23% of all patients). If the ALL and ANLL patients of the present study were combined we would arrive at conclusions similar to those of Spitzer et al (1976). Knudtzon (1977) reported that out of 50 patients with AML, 16 ALL, 10 CML and 5 lymphomas only 38 of the AML patients were evaluable and the treatment was highly variable. Nevertheless, there is an agreement with our results that growth of more than 1000 cell aggregates show the poorest prognosis. Agreement is also found when comparing the present study with that of Beran et al (1980) (26 patients) where 'extensive growth' showed a poorer response to therapy, whereas the 'low growth' category (includes mostly group A and B in our study) had the best prognosis.

Discordant conclusions are drawn from our results and the study of Vincent et al (1977) (43 patients), who found a higher remission frequency (65%) in patients with no growth in agar compared with growth in vitro (30%). These authors, however, scored their cultures on day 14 only at a time when most small clusters have disintegrated. Some of the discrepancies between the present and previous studies may also be due to different culture conditions such as cell separation techniques and sources of colony stimulating activity.

In general, the selection of patients may also play an role as well as divergent treatment of the patients. In the present study the distinction was made between ANLL and ALL patients. Patients with preleukaemia or leukaemia secondary to treatment of

other malignancies were excluded as well as untreated ANLL patients in order to make the patient material as homogeneous as possible. Previous studies have included several different treatments, e.g. in the study of Moore et al (1974) 6 different regimes were employed. In the present report principally 2 different regimes were used. If the growth pattern of the 33 TRAP-treated patients is analyzed separately, however, there is still a significant difference in the remission rate between group A + B compared to group C + D. In the 20 patients treated with prednimustine only 2 patients achieved complete remission, 1 patient in each of group A and B. It should be noted that prednimustine-treated patients, in all 4 groups, had a 10 year higher median age than the corresponding growth pattern groups of TRAP-treated patients, which may partly explain the lower remission frequency. In addition, even if the 6 patients who did not survive for more than 1 month are excluded the prognostic significance of the growth pattern is still significant.

Another factor with implications for the prognosis was the myeloblast labelling index. Taken as such, the labelling index was not a very strong indicator but in combination with the growth pattern it was useful; all patients in groups A and B with a LI below 15 achieved complete remission. Earlier studies using the myeloblast LI to evaluate prognosis have been contradictory (Crowther et al 1975, Hart et al 1977). The shorter survival time noticed in patients with a high LI, however, is in accordance with the results of Crowther et al (1975) and Hart et al (1977). This observation may reflect a faster rate of growth of the leukaemic cell population in these patients.

In agreement with the reports by Vincent et al (1977) and Beran et al (1980) we found

a fairly good correlation between the growth pattern of clonogenic cells in marrow and blood. This was in contrast to the results of Tebbi et al (1976).

The clonogenic cells in AML often have a lighter density than normal (Moore et al 1973, 1974), and this was found also to be true in the present study. The number of patients was, however, too small to allow any prognostic conclusions to be made from the proportion of cluster forming cells lighter than 1.062 g/ml.

The overall conclusion is that the *in vitro* growth pattern at diagnosis is a prognostic measure per se and an indicator of which patients will respond poorly to current therapy. This does not mean that other factors are without importance for the prognosis, e.g. age and intensity of treatment. A good prognosis in terms of remission rate did not automatically imply a long survival. Among the TRAP-treated patients group A showed a complete remission 10/13 patients and a median survival time of 7.4 months compared to group B with 9/9 remissions and a median survival time of 16.6 months. Conversely, 2 of the 3 patients in group C and D entering remission survived for more than 3 years. It seems, therefore, that the *in vitro* growth pattern has stronger prognostic implications with regard to remission rate than survival time.

The ALL patients studied for comparison were all either group A or group D and there was no difference in remission frequency between these 2 groups. Although the ALL patients were few, it was possible to conclude that the *in vitro* growth pattern of marrow cells is of limited prognostic value in ALL.

It is an important question whether the colonies/clusters growing *in vitro* are derived from leukaemic cells or from residual

normal stem cells. There is, however, no simple way of determining the origin of colonies and clusters. Evaluation of the differentiation capacity in agar cultures has shown defective maturation in colonies and clusters in acute leukaemia (Moore et al 1973, 1974, Vincent et al 1977), indicating a leukaemic origin of the clonogenic cells. Karyotypic anomalies which are found within colonies grown from patients with acute leukaemia point in the same direction (Dut-tera et al 1972, Moore & Metcalf 1973). But there is still the possibility that some of the colonies and clusters develop from normal stem cells. It is noteworthy that the growth patterns that are closest to the normal, i.e. group A and B, also showed the highest remission frequency. Although not proven by the present study, some of the colonies and clusters growing in group A and B may represent residual normal stem cells necessary for repopulation of the marrow during remission induction. Sequential studies of bone marrow *in vitro* growth in ANLL during remission induction and remission have shown striking oscillations of the colony and cluster formation possibly reflecting a competition between normal and leukaemic cells upon chemotherapeutic perturbation, as shown previously for 8 of the patients in this study (Gustavsson et al 1979).

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The relationship between growth in agar, karyotype and prognosis in acute leukaemia

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The growth pattern in agar culture and the karyotype of bone marrow cells were studied in 79 patients with untreated acute non-lymphocytic leukaemia (ANLL). Results were divided into the following groups: (A) colony and cluster formation; (B) growth of less than 600 small clusters per 10^5 cells; (C) growth of more than 600 small clusters; (D) no growth in agar. Cytogenetically, the patients were divided into 3 categories: NN, normal metaphases only; AN, both abnormal and normal metaphases and AA, abnormal metaphases only. An association was seen between growth pattern and karyotype: the majority of NN patients (33/37) belonged to group (A+B) while in group (C+D) 20/24 patients were AN or AA. 37 patients were prognostically evaluable. The growth pattern in agar but not the cytogenetic pattern had prognostic implications. 25 patients with acute lymphocytic leukaemia (ALL) were also studied at diagnosis. Different growth patterns in agar had no impact on prognosis. No relationship was detected between growth pattern and karyotype in ALL.

Key words: acute leukaemia – agar culture – karyotype

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The in vitro growth pattern in agar of bone marrow cells from patients with acute leukaemia is variable and most often abnormal. Colony formation (> 40 cells) is usually absent but various numbers of clusters (3–40 cells) develop or there is no growth at all (1–3). Several studies have compared the in vitro growth pattern with prognostic outcome (3–12). Differences in culture technique, sources of colony stimulating activity (CSA), classification of growth pattern and therapy make it difficult to compare the results, but several common features can be identified. For instance,

growth of colonies and clusters is usually combined with a favourable prognosis (3, 11) whereas no growth or an excessive production of only small clusters is associated with poor prognosis (3, 8, 10, 11). But prognostic implications of growth pattern in vitro of acute lymphocytic leukaemia (ALL) have not been found (8, 11–13).

Cytogenetic studies of bone marrow cells have shown that numerical and/or structural abnormalities occur in approximately 50% of patients presenting with acute leukaemia (14–17). Although the karyotypic changes may be variable, there is a

strong tendency for a non-random chromosome involvement. Several studies have shown that the karyotypic pattern in ANLL is correlated with survival and that patients with a normal karyotype (NN) have a longer median survival time than patients with a mixture of normal and abnormal karyotypes (AN) or totally abnormal karyotypes (AA) (14, 18-21). Furthermore, in ALL, the karyotype is a prognostic factor in both children and adults (16, 17).

To extend previous studies, we compared in 79 patients with acute non-lymphocytic leukaemia (ANLL) and in 25 patients with ALL the *in vitro* growth pattern in agar of marrow cells with results from karyotype analyses.

MATERIAL AND METHODS

Patients and treatment

From 1975 to 1981, 79 unselected patients with acute non-lymphocytic leukaemia (ANLL) were studied at diagnosis. They were classified according to the criteria suggested by the FAB co-operative study group (22). Their ages ranged from 15 to 84 years with a median age of 50 years. There were 37 females with a median age of 51 years and 42 males with a median age of 47 years.

11 patients had ANLL secondary to radiotherapy and/or chemotherapy for another malignancy; 2 patients with Hodgkin's disease, 2 with non-Hodgkin lymphoma, 3 with polycythaemia vera, 3 with gynecologic malignancy and 1 patient with breast cancer. 6 patients had a preleukaemic syndrome terminating in ANLL. Patients with secondary leukaemia and those with a preleukaemic syndrome terminating in leukaemia were excluded from the prognostic evaluations.

62 patients were classified as *de novo* ANLL. 37 of these patients with optimal chemotherapy (denoted with an asterisk in Table 1) were evaluated in the prognostic estimates. The induction treatment was in 34 patients TRAP: cytosine arabinoside (Ara-C) *i.v.*, thioguanine and prednisone orally for 5 d with daunorubicin (DNR) *i.v.* on the first day. 2 patients were treated with only Ara-C and DNR, and 1 patient had COAP-therapy: cyclophosphamide *i.v.*, Ara-C *i.v.* and prednisone orally for 5 d with vincristine *i.v.* on the first

day. The treatment was repeated if possible every 10th day. Complete remission (CR) was achieved in 27/37 patients (73%). CR was defined as normal bone marrow cellularity with less than 5% blast cells, return of neutrophil counts to $1.5 \times 10^9/l$ or greater, and platelet counts to at least $100 \times 10^9/l$. Survival time was calculated from the day of diagnosis. Maintenance therapy consisted of TRAP twice, alternated with COAP twice, with one cycle each month. 25 patients with ALL were also studied. There were 9 females with a median age of 52 years and 16 males with a median age of 32 years. No patient had been treated earlier with chemo- or radiotherapy.

In vitro culture

Bone marrow cells were collected in heparinized McCoy's medium, separated in Isopaque-Ficoll (1.077 g/ml) and the cells from the interphase cultured at 1×10^5 cells/ml in 0.3% agar on top of a feeder layer containing 3×10^5 blood mononuclear cells from a single donor. Small clusters (3-20 cells) were scored on day 7 and colonies (more than 40 cells) and large clusters (21-40 cells) on day 10 of incubation at 37°C in 5% CO₂ in humidified air.

We distinguished 4 different growth patterns.

Group A: growth of colonies (> 40 cells) and clusters (3-40 cells).

Group B: no growth of colonies but less than 600 small clusters (3-20 cells) per 10^5 cells.

Group C: no growth of colonies, but more than 600 small clusters per 10^5 cells.

Group D: no growth of either colonies or clusters.

Only a minority of the patients showed any growth of large clusters. These patients were included in either group A or B depending of the growth of colonies or small clusters, because taking large cluster growth into consideration was of no prognostic value (data not shown). We have found that patients belonging to groups A and B have a more favorable prognosis with a higher remission frequency than patients within groups C and D (11). Therefore, in the prognostic evaluation, groups A and B (A+B) and groups C and D (C+D), were considered together.

Chromosome analysis

The chromosomes were studied at the time of diagnosis in direct bone marrow preparations or after short-term (12-20 h) cultures of marrow cells, or a combination of these methods. The chromosomes

were stained by means of the trypsin-Giemsa banding technique. The preparation and staining methods have been described previously (23). Karyotype analyses were made by photography, each karyotype being checked in addition directly in the microscope. The chromosomes were classified according to the criteria recommended by the ISCN (24). At least 2 cells with the same extra chromosome or structural rearrangement, or 3 cells with the same missing chromosome were required for recording a chromosomal aberration. The patients were divided into 3 groups according to the presence or absence of cells with chromosome abnormality: NN, normal metaphases only; AN, normal and abnormal metaphases; AA, abnormal metaphases only.

Statistical analysis

Two-sided χ^2 and Fisher's exact tests were used. In the prognostic estimates the interactions between different factors have been evaluated multivariately as a multidimensional contingency table analysis. The median survival was calculated using the life-table technique.

RESULTS

Growth pattern and cytogenetic pattern in ANLL

Basic information on the patients as well as results from in vitro growth and karyotype analyses are given in Table 1. Figure 1 illustrates the relationship between cytogenetic pattern and in vitro growth pattern of all 79 patients studied. The frequency of NN-patients in group (A+B) (33/55) was higher than in group (C+D) (4/24) ($P < 0.001$). Conversely, the incidence of AA-patients was higher in group (C+D) (12/24) than in group (A+B) (10/55) ($P < 0.005$). The frequency of AN-patients did not differ between group (A+B) (12/55) and (C+D) (8/24). Thus, these data show a clear association between growth pattern and karyotype in ANLL. Cytogenetic analysis of 4 additional patients failed because no mitoses were found. The bone marrow cells of 3 of these patients belonged to group D (no

growth in agar), while the 4th patient belonged to group A.

The 42 patients with abnormal karyotypes had a total of 109 aberrations. The following non-random changes were noted: $-5/5q-$, i.e. monosomy 5 or deletion of the long arm of chromosome 5 (7 patients); $-7/7q-$, i.e. monosomy 7 or deletion of the long arm of chromosome 7 (9 patients); $+8$, i.e. trisomy 8 (6 patients); $t(8;21)(q22;q22)$, i.e. a translocation of the distal segment of chromosome 8 to chromosome 21 (4 patients); $t(9;22)(q34;q11)$, i.e. a Philadelphia chromosome with the typical 9;22 translocation (3 patients); and $t(15;17)(q22;q21)$, i.e. a reciprocal translocation of the distal segments of chromosomes 15 and 17 (1 patient). Altogether, 26 of the 42 patients with aberrations had at least 1 of these characteristic aberrations. 1 additional patient (case no. 63) had $i(\text{del}(17)(q21))$, probably originating from a $t(15;17)$ by a misdivision of the deleted chromosome 17; the segment translocated to chromosome 15 could, however, not be unequivocally determined.

Figure 2 shows the distribution of all chromosomes involved in aberrations in relation to growth pattern in agar. The chromosomes involved in the non-random changes characteristic of ANLL, i.e. chromosomes 5, 7, 8, and 21, were in general similarly engaged in the 4 groups. However, a striking difference was observed between group (A+B) versus group (C+D); in group (A+B) (22 patients), chromosome 3 was engaged in aberrations in 7 patients and chromosome 12 in 9 patients, whereas none of the 20 patients in group (C+D) displayed an engagement of any of these chromosomes. It may also be mentioned that all 4 structural aberrations of chromosome 3 affected the short arm, and all breakpoints were located within the adjacent bands

TAB
Data on 79 patients with ac

Case no.	Sex/age	Growth pat- tern	Cyto- genetic pat- tern	Karyotype	No. of mito- ses	Treatment	Remis- sion	Surviva- l (days)
1*	F /19	A	NN	46,XX	21	TRAP	CR	263
2*	M/20	A	NN	46,XY	21	TRAP	CR	461
3	M/36	A	NN	46,XY	18	PM+VCR	CR	299
4*	M/38	A	NN	46,XY	9	TRAP	CR	873
5*	M/46	A	NN	46,XY	20	TRAP	CR	686
6*	M/49	A	NN	46,XY	50	TRAP	CR	171
7*	M/52	A	NN	46,XY	33	TRAP	CR	426
8*	M/59	A	NN	46,XY	25	TRAP	CR	206
9	F /64	A	NN	46,XX	33	PM+VCR	NR	76
10	F /71 ^{P1}	A	NN	46,XX	40	0	-	124
11	F /75 ^S	A	NN	46,XX	200	0	-	271
12	M/15	A	NN	46,XY	25	PM+VCR	NR	52
13*	M/24	A	NN	46,XY	19	TRAP	NR	222
14*	M/47	A	NN	46,XY	25	TRAP	NR	205
15	M/62 ^{P1}	A	NN	46,XY	13	PM	NR	335
16	M/75 ^{P1}	A	NN	46,XY	16	0	-	297
17	M/80	A	NN	46,XY	5	0	-	127
18	F /43 ^S	A	AN	46,XX/47-48,XX,+9,-18,+del(6)(q16),+2mar	25	TRAP	CR	175
19*	M/26	A	AN	46,XY/46,XY,t(9;22)(q34;q12)	108	TRAP	CR	296
20*	M/30	A	AN	46,XY/45,X,-Y	34	TRAP	CR	216
21*	F /38	A	AN	46,XX/46,XX,t(9;22)(q34;q12)/46-48,XX,t(9;22),-3,+8,+13,dup(1)(q31;q44).inv(2)(p15;q11),+del(22)(q12)	21	TRAP	NR	260
22*	F /63	A	AN	46,XX/44,XX,-7,-20,del(3)(p21),del(5)(q12),i(22q)	40	TRAP	NR	56
23	F /74	A	AN	46,XX/48,XX,+3,+8	21	PM	NR	24
24*	M/18	A	AN	46,XY/46,XY,t(1;12)(p36;p12)	19	TRAP	NR	308
25*	F /69	A	AA	39,X,-X,-3,-5,-13,-14,-15,-16,-17,-21,-22,del(7)(p11),12p+,16q+,+3mar	30	COAP	CR	244
26*	M/43	A	AA	46,XY,del(7)(q32)	42	TRAP	CR	1023
27	F /64 ^S	A	AA	47,XX,+12	3	VCR+TG+Ara-C	NR	147
28	F /74	A	AA	48,XX,-5,+10,+11,+21,t(1;3)(q44;p14).del(3)(p14;q21)	25	0	-	?
29	M/57 ^S	A	AA	44,XY,-9,-12,del(3)(p22),17p+	25	TRAP	NR	25
30	M/65 ^S	A	AA	45,XY,-7	48	0	-	196
31*	F /21	B	NN	46,XX	25	TRAP	CR	528
32*	F /26	B	NN	46,XX	25	TRAP	CR	404
33*	F /34	B	NN	46,XX	46	TRAP	CR	839
34*	F /39	B	NN	46,XX	25	Ara-C+DNR	CR	251
35*	F /40	B	NN	46,XX	25	TRAP	CR	499
36*	F /44	B	NN	46,XX	23	TRAP	CR	1485
37*	M/20	B	NN	46,XY	25	TRAP	CR	604
38*	M/38	B	NN	46,XY	25	TRAP	CR	606
39*	M/41	B	NN	46,XY	25	TRAP	CR	587
40*	M/44	B	NN	46,XY	25	TRAP	CR	384
41	F /56 ^{P1}	B	NN	46,XX	39	0	-	227
42	M/48	B	NN	46,XY	20	PM+VCR	NR	158

nonlymphocytic leukaemia^{a)}

Case no.	Sex/age	Growth pattern	Cyto-genetic pattern	Karyotype	No. of mitoses	Treatment	Remission	Survival (days)
43	M/61	B	NN	46,XY	25	PM	NR	7
44	M/66	B	NN	46,XY	30	Ara-C+DNR	NR	6
45	M/72 ^{P1}	B	NN	46,XY	46	PM	NR	112
46	M/82	B	NN	46,XY	25	0	-	16
47*	M/38	B	AN	46,XY/46-50,XY,+Y,-8,+9,+17,+22,+del(7)(p11),del(12)(p11),21q+	50	TRAP	CR	559
48	F/51	B	AN	46,XX/45,XX,-7	16	PM+VCR	NR	168
49	F/54 ^{P1}	B	AN	46,XX/45,XX,-12,t(3;6)(p23;p25),del(5)(q13),i(17q)	50	0	-	80
50	F/64	B	AN	46,XX/42-44,XX,+8,-12,-13,-14,t(2;5)(q12;q35)	24	PM	NR	32
51	M/55	B	AN	46,XY/46-47,XY,+8,-17	50	PM+VCR	NR	130
52*	M/16	B	AA	45,X,-Y,t(8;21)(q22;q22),+21q+	28	TRAP	CR	437
53	F/70 ^S	B	AA	45,XX,-7,12q+	26	0	-	122
54	M/57	N	AA	47,XY,+19,del(4)(q26),del(5)(p12),t(7;12)(q21;p13)	25	0	-	76
55	M/82	B	AA	45,X,-Y,t(8;21)(q22;q22)	5	0	-	38
56	F/65	C	NN	46,XX	11	PM	NR	88
57	M/54	C	NN	46,XY	70	0	-	19
58*	F/27	C	AN	46,XX/47,XX,+22	17	Ara-C+DNR	NR	14
59*	F/41	C	AN	46,XX/45,X,-X,t(8;21)(q22;q22)	19	TRAP	NR	121
60	F/68	C	AN	46,XX/47,XX,+min mar	25	PM	NR	43
61	M/32	C	AN	46,XX/44-45,XY,+2,-5,-7	25	PM+VCR	NR	128
62	M/67 ^S	C	AN	46,XY/47-48,XY,+8,+9	34	0	-	42
63*	F/30	C	AA	46,XX,i(del(17)(q21))	25	TRAP	CR	469+
64*	M/38	C	AA	46,XY,t(15;17)(q22;q21)	30	TRAP+BMT	CR	635+
65*	F/23	C	AA	47-48,XX,+10,t(11;17)(q23;25)	28	TRAP	CR	270
66*	F/15	C	AA	46,XX,t(8;16)(p11;q13)	31	TRAP	NR	8
67	F/42 ^S	C	AA	46,XX,11q+	50	TRAP	NR	68
68	F/50	C	AA	46,X,del(X)(q21),t(5;21)(q13;q22)	36	PM+VCR	NR	118
69	F/53	C	AA	46,XX,t(8;21)(q21;q22)	20	PM+VCR	NR	43
70	M/32 ^S	C	AA	46,XY,21q+	50	Ara-C+TG+A+Mtx+P	NR	109
71*	M/53	D	NN	46,XY	25	TRAP	CR	353+
72*	F/51	D	NN	46,XX	9	TRAP	NR	182
73	F/44 ^S	D	AN	46,XX/45,XX,-7	25	TRAP	CR	1902+
74	F/73	D	AN	46,XX/47,XX,+11	12	0	-	204
75	F/84	D	AN	46,XX/47,XX,+del(9)(q13)	37	PM	NR	43
76	F/63	D	AA	45,XX,-7/46,XX,-7,+mar	23	PM+VCR	NR	17
77	M/26 ^S	D	AA	46,XY,18q+	25	0	-	36
78*	M/63	D	AA	46,XY,t(9;22)(q34;q12)	25	TRAP	NR	192
79	M/84	D	AA	42-45,XY,-5,-5,+8,-14,-15,-17,-18,-20,+3mar	35	PM	NR	30

Growth pattern, cytogenetic pattern and treatment with TRAP and COAP are detailed in Material and Methods. PM, prednimustine; VCR, vincristine; Ara-C, cytosine arabinoside; DNR, daunorubicin; TG, thioguanine; A, adriamycin; Mtx, methotrexate; P, prednisone; BMT, bone marrow transplantation; +, patients still alive; CR, complete remission; NR, no remission; P1, previous preleukaemic phase; S, secondary leukaemia. Patients denoted with an asterisk are included in prognostic evaluation.

a) The karyotypic aberrations in 25 patients have been reported previously: case no. 62 (33); case no. 70 (34), and case nos. 19, 20, 22, 23, 26, 28, 30, 47-51, 55, 58, 59, 61, 65, 66, 68, 69, 73 and 75 (15).

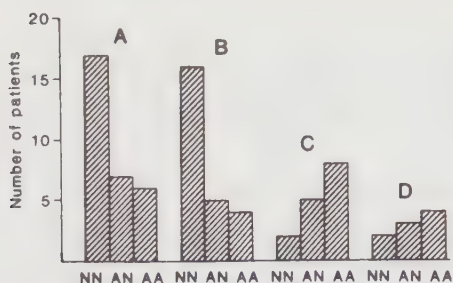


Figure 1. Distribution of the 3 cytogenetic patterns NN, AN and AA among the 4 growth pattern groups A, B, C and D, in the 79 patients with acute non-lymphocytic leukaemia. NN, all metaphases normal; AN, mixture of normal and abnormal metaphases; AA, all metaphases abnormal. Group A, growth of colonies and clusters; group B, no colonies and less than 600 small clusters per 10^5 cells; group C, no colonies but more than 600 small clusters per 10^5 cells; group D, no growth of colonies or clusters.

p14-p23. Also, 4 of 5 structural changes of chromosome 12 affected the short arm. 2 of the 3 numerical aberrations of chromosome 3, and 3 of the 4 numerical aberrations of chromosome 12 were monosomies. Thus, it is suggestive that 6 of the 7 aberrations affecting chromosome 3, and 7 of the 9 aberrations affecting chromosome 12 resulted in a loss of genetic material from the short arms of these chromosomes.

The incidence of non-random and specific aberrations in relation to the growth pattern is presented in Table 2. 16 of the 22 patients with chromosomal aberrations in group (A+B) had at least 1 of these changes and 10 of 20 patients in group (C+D) ($P > 0.10$). Thus, no apparent differences were noted among the 4 growth pattern groups as regards the distribution of non-random and specific chromosome aberrations.

Complex chromosome aberrations were predominantly found in patients with growth pattern A and B, whereas patients in groups

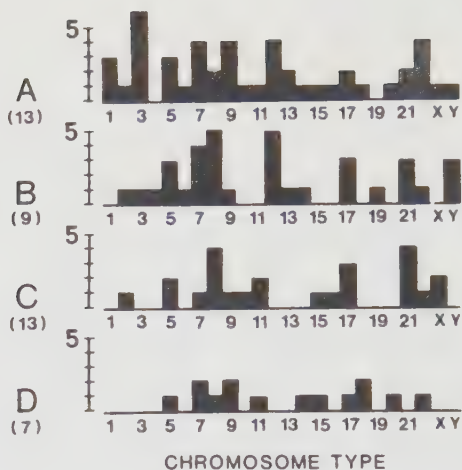


Figure 2. Distribution of the chromosomes involved in aberrations in relationship to in vitro growth patterns. The ordinate shows the number of patients. Numbers in brackets show the number of patients in each growth pattern group (A, B, C and D).

C and D were characterized by less complex changes (Table 3). Thus, 11 of 22 patients in group (A+B) had 3 or more aberrations per patient as compared to only 2 of 20 patients in group (C+D) ($P < 0.005$).

11 patients had developed ANLL after treatment for another malignancy. All 4 growth patterns were represented (5A, 1B, 3C and 2D). 10 of the 11 patients had chromosome aberrations. This number of patients is too small to permit any conclusions with regard to the relationship between growth pattern and cytogenetic pattern.

Growth and cytogenetic pattern in ALL

Clinical data on the 25 ALL-patients and results from in vitro growth and karyotype analyses appear in Table 5. No patient had growth pattern C. Patients with and without abnormal metaphases were evenly distributed between group (A+B) and group D. In group (A+B) there were 4 NN-, 2 AN- and 3 AA-patients compared to 5 NN-, 2 AN-

TABLE 2
Incidence of non-random and specific aberrations among AN and AA patients with ANLL

Growth pattern	No. of patients	Type of aberration						Total incidence (patients)
		-5/5q-	-7/7q-	+8	t(8;21)	t(9;22)	t(15;17)	
Group A	13	3	3	2	0	2	0	8
Group B	9	1	3	2	2	0	0	8
Group C	13	2	1	1	2	0	1	6
Group D	7	1	2	1	0	1	0	4
Total	42	7	9	6	4	3	1	26

TABLE 3
Number of chromosome aberrations per patient in relation to growth pattern; ANLL-patients

Growth pattern	No. of aberrations				No. of patients
	1	2	3	>3	
Group A	6	1	-	6	13
Group B	1	3	1	4	9
Group C	8	4	1	-	13
Group D	4	2	-	1	7
Total	19	10	2	11	42

and 5 AA-patients in group D. Thus, there was no relationship between growth pattern in agar and karyotype. In 4 patients, cytogenetic analysis failed because no mitoses were found. The marrow cells of 3 of these patients did not grow in agar.

The karyotypes were evaluable in 21 patients. Chromosomal abnormalities were found in 12/21 (57%) patients. 6 patients had 1 of the 3 aberrations reported as typical or ALL: 4 patients showed t(9;22) and 1 patient each had t(4;11) and t(8;14). One additional patient (case 5) with a missing chromosome 8 had a 14q+ marker chromosome, morphologically indistinguishable from the t(8;14). Only 2 of these 7 patients showed growth of marrow cells in vitro. The number of patients is too limited in this study to relate specific aberrations to growth pattern.

Growth pattern, cytogenetic pattern and prognosis

The prognosis was evaluated in 37 ANLL-patients considered to have received optimal chemotherapy (Tables 1 and 4); patients treated in the early part of this study with prednimustine or prednimustine and vincristine were excluded from prognostic evaluations since only 1/17 achieved complete remission. It is concluded that this type of chemotherapy is not optimal in treatment of ANLL and prognostic evaluations are therefore not meaningful for patients subjected to this treatment regimen. However, PM-treated patients were not different from other patients in terms of growth pattern or cytogenetic pattern, but the median age was higher, 61 years (range 15-84).

TABLE 4
Growth pattern and cytogenetic pattern in relation to survival in ANLL-patients

Cyto-genetic pattern	Growth pattern A + B					Growth pattern C + D					All patients				
	Pts	Age	CR	Survival (mo)	Alive >1 yr	Pts	Age	CR	Survival (mo)	Alive >1 yr	Pts	Age	CR	Survival (mo)	Alive >1 yr
NN	19	39	17	15.4	13	2	52	1	8.9	0	21	40	18	14.2	13
AN	6	34	3	9.3	1	2	34	0	2.3	0	8	34	3	8.0	1
AA	3	43	3	14.6	2	5	30	3	9.0	2	8	34	6	14.0	4
Total	28	38	23	14.0	16	9	38	4	6.7	2	37	38	27	11.8	18

The table shows the number of patients (Pts), their median age, complete remissions (CR), median survival in months and the number of patients alive after more than 1 year.

The remission frequency in group (A+B) was 82 % (23/28) and thus higher than in group (C+D), 44 % (4/9) ($P = 0.04$). Patients with normal metaphases (NN) had remission frequency, 86 % (18/21) and patients with abnormal metaphases (AN or AA), 56 % (9/16); this difference was not significant ($P = 0.06$). A multivariate analysis of the influence of age showed no correlation with remission frequency ($P = 0.3$), cytogenetic pattern ($P = 0.5$) or growth pattern ($P = 0.7$).

Table 4 shows the survival data for all the 37 patients evaluated. Life-table analysis showed median survival for patients with growth pattern (A+B): 14 months compared with 6.7 months in (C+D) ($P = 0.07$). NN-patients with median survival 14.2 months differed from AN-patients with 8 months survival ($P = 0.02$), while there was no difference from AA-patients; 14 months.

In ALL-patients, remission frequency in group (A+B) was 7/10 and median survival 9.4 months compared with remission rate in group D 7/15, and median survival 8.6 months. Thus, there was no relation between growth pattern and remission ($P = 0.17$) or survival time ($P > 0.5$). In addition, we did not find any correlation between WBC counts and growth pattern, cf. Table 5. Patients with normal metaphases (NN) had remission frequency 5/9 and median survival 9.7 months, while 5/12 patients with abnormal metaphases (AN and AA) achieved complete remission, with median survival time 6.3 months. No correlation between karyotypic pattern and remission rate ($P = 0.28$) or survival time ($P > 0.1$) was found.

Data on 25 patients with acute lymphocytic leukaemia

Case no.	Sex/Age	Growth pattern	Cyto-genetic pattern	Karyotype	No. of mitoses	WBC	Induction treatment	Remission	Survival (days)
1	M/21	A	NN	46,XY	12	19.9	PM+VCR	CR	305
2	M/35	A	NN	46,XY	25	17.3	COAP	CR	202
3	M/13	A	AN	46,XY/46,XY,+1,-8,t(14;?) (q32;?)	44	27.6	A+VCR+P+Mtx it, XRT	CR	258+
4	M/68	A	AN	46,XY/47-51,XY,+7,+9,+del(3) (q11),t(6;19)(q15;q13),+2mar	29	3.5	PM+VCR	NR	39
5	M/23	A	AA	47-48,XY,+1-2mar	25	6.0	PM+VCR	NR	68
6	F/28	A	AA	48,XX,+19,+mar	26	107	A+VCR+P+Mtx it, XRT	CR	538+
7	M/21	B	NN	46,XY	44	35.3	A+VCR+P+Mtx it, XRT	CR	323
8	F/21	B	NN	46,XX	14	12.9	A+VCR+P+Mtx it	NR	18
9	F/57	B	AA	46,XX,t(9;22)(q34;q11)	22	213	A+VCR+P	CR	499+
10	F/60	B	-	failure	0	3.4	PM+VCR → TRAP	CR	510+
11	M/17	D	NN	46,XY	25	188	A+VCR+P+Mtx it, XRT → TRAP → COAP	NR	291
12	M/22	D	NN	46,XY	18	3.1	A+VCR+P+Mtx it, XRT	CR	105+
13	M/23	D	NN	46,XY	25	1.9	A+VCR+P+Mtx it, XRT → PM → L-aspar	NR	958
14	M/43	D	NN	46,XY	18	4.3	TRAP	CR	470
15	F/52	D	NN	46,XX	25	217	A+VCR+P → TRAP	NR	121
16	M/60	D	AN	46,XY/56,XY,t(9;22)(q34;q11),+4, +5,+8,+11,+16,+17,+18,+19,+20	29	19.2	PM+VCR → A+VCR+ P+Mtx it → TRAP	NR	248
17	F/71	D	AN	46,XX/46,XX,t(9;22)(q34;q11)	31	13.5	PM+VCR	NR	74
18	F/26	D	AA	50-51,XX,+4-5mar	14	36.5	A+VCR+Ara-C+P+Mtx it, XRT	CR	1082
19	M/29	D	AA	46,XY,t(4;11)(q22;q25)	23	169	A+VCR+P+Mtx it, XRT	CR	258
20	M/59	D	AA	47,XY,+10	18	14.1	A+VCR+P → TRAP	NR	127+
21	M/62	D	AA	46,XY,t(8;14)(q23;q32)	63	10.3	PM+VCR	NR	50
22	M/69	D	AA	46,XX,t(9;22)(q34;q11)	5	182	PM+VCR	NR	60
23	F/23	D	-	failure	0	1.9	TRAP	CR	835
24	F/51	D	-	failure	0	46.5	A+VCR+P+Mtx it	CR	512
25	M/60	D	-	failure	0	1.3	COAP	CR	617

Growth pattern, cytogenetic pattern and treatment with TRAP and COAP are detailed in Materials and Methods.
Colony and cluster growth (mean and range): Group A, 10; 4-22 colonies; 48; 16-90 clusters. Group B, 36; 17-75 clusters.
PM, prednimustine; VCR, vincristine; A, doxorubicin; P, prednisone; Mtx, methotrexate; L-aspar, L-asparaginase; Ara-C, cytosine arabinoside; XRT, cranial radiotherapy; it, intrathecal; +, patient still alive; CR, complete remission; NR, no remission.

DISCUSSION

Both the *in vitro* growth pattern and the karyotype of marrow cells have been claimed to be of prognostic significance in ANLL (see introductory section for references). To our knowledge, the present report is the first comparing both these measurements in relation to prognosis. Our objectives were 2-fold: to determine whether any relationship existed between *in vitro* growth pattern and karyotype and to determine whether a combination of these measurements improved the prognostic prediction.

We found a striking association between growth in agar and karyotype, inasmuch as patients with normal metaphases only (NN) preferentially showed growth pattern A or B. In contrast, patients with growth patterns C and D predominantly showed karyotypes AN or AA. The incidence of aneuploidy is probably underestimated. First, patients (less than 10% of the total) with abnormal clones that could not be defined by banding were excluded. The incidence of excluded patients corresponds well with other recently published series of patients with ANLL (25). Second, bone marrow samples from many patients were studied only in direct preparations. Some patients considered to be normal on the basis of a directly-processed sample may have an abnormal clone in short-term cultures (26–28). Third, most of the patients were analyzed with a technique that does not yield an adequate number of cells with elongated prometaphase chromosomes. Subtle rearrangements, such as those recently reported by Yunis et al (29), may therefore have been overlooked. The fact that chromosome abnormalities present were in 20 of 24 patients in growth pattern group (C+D), in contrast to only 22 of 55 patients in group

(A+B), would then indicate that such minor subtle changes, not detectable by the use of conventional direct bone marrow preparation techniques, have been underestimated preferentially in patients of groups A and B.

Among patients with chromosomal abnormalities, the non-random aberrations were evenly distributed in different growth pattern groups. However, those patients within groups A and B displaying abnormal metaphases had more complex chromosome aberrations than those patients within groups C and D. A higher frequency of patients with abnormal chromosomes in group (C+D) compared to group (A+B) suggests a more advanced disease in the former group consistent with the worse prognosis found. However, the chromosomal aberrations in group (C+D) although more frequent, were less complex than those in group (A+B). One explanation for this finding is that clonal evolution has worked longer or faster in group (C+D) to select more homogenous cell populations with a growth advantage. Sequential studies to look for a drift from groups A and B to groups C and D with time would be informative.

It is an interesting observation but not fully understood that chromosomes 3 and 12 were involved in aberrations only in groups A and B. Although patients with involvement of either chromosome 3 or 12 showed a prognostically favourable growth pattern, a good prognosis was not implied inasmuch as only 2 of 9 such patients receiving chemotherapy achieved remission.

The results from agar growth of marrow cells in ALL showed either sparse growth of cell aggregates or no growth at all. However, there was no difference in remission frequency or survival time between these 2 groups

consistent with results of previous studies (6, 8, 12, 13). The karyotypic findings in ALL agree with results in previous reports, but the number of patients is too limited in this study for detection of prognostic implications of specific aberrations in ALL which have been documented previously (16, 17). An observation perhaps worth mentioning is that the 4 patients with no evaluable mitoses all attained complete remission and had a remarkably long median survival; 19 months compared with 8.6 months for the whole patient material. Leukaemic cells of these patients may have a small growth fraction, which could be the reason why no mitoses were found, and also possibly contribute to the long survival of the patients.

We do not know for individual patients whether clonogenic cells in leukaemia actually have the same karyotype as other marrow cells. There are, however, reports showing that colonies and clusters in leukaemia have the same karyotype as mixed marrow cells (30–32). The growth pattern in patients with (AN) depends on the relative plating efficiency of clones with and without chromosomal abnormalities.

We conclude that there is a relationship in ANLL between growth pattern and cytogenetic pattern despite the extreme variability of karyotypes compatible with almost all types of growth pattern. In relation to remission and survival, the growth pattern was of prognostic significance, whereas the cytogenetic pattern per se did not show any relationship to prognosis. This finding is in contrast to earlier reports (14, 18–21), that showed a longer median survival for NN-patients than AN- or AA-patients. However, in these reports, all treated patients were included, whereas in the present work only patients considered to be optimally treated were included in the analysis

of survival. If all treated patients of our present study are considered, the median survival times are 10.6 months for NN-patients, 4.3 months for AN-patients and 4.4 months for AA-patients, which agrees very well with the previous studies cited above. In contrast to ANLL, we did not find any relationship between growth pattern in agar and the presence or absence of chromosomal abnormalities in ALL.

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PREDICTION OF RESPONSE TO CHEMOTHERAPY IN ACUTE LEUKEMIA BY IN VITRO
DRUG SENSITIVITY TESTING ON LEUKEMIC STEM CELLS¹

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3. The abbreviations used are: ara-C, 1- β -D-arabinofuranosylcytosine; 6-TG, 6-thioguanine; TRAP, thioguanine, daunorubicin, cytosine arabinoside and prednisone; COAP, cyclophosphamide, Oncovin, cytosine arabinoside and prednisone; LD₅₀, the drug concentration causing 50 per cent reduction of clonogenic cells; AUC, area under the curve; CR, complete remission; NR, no remission; RAEB, refractive anemia with excess of blasts; FCS, fetal calf serum.

ABSTRACT

Twenty patients with acute myeloid leukemia were studied for in vitro drug sensitivity of the leukemic clonogenic cells in agar. The cells were preincubated for one hour with ara-C (0.15, 0.3, 0.6, 1.2 and 2.4 $\mu\text{g/ml}$) or daunorubicin (18, 37, 75, 150, and 300 ng/ml), washed and plated in agar and cluster/colonies counted after 10 days incubation. Survival curves were constructed and the area under the curve (AUC) used for calculation of the surviving fraction of clonogenic cells. In seventeen patients treated with TRAP the in vitro drug sensitivities could be correlated to the in vivo response to therapy. Patients who entered remission (11/17) were significantly more sensitive to ara-C ($p < 0.005$) and to daunorubicin ($p < 0.02$) than patients who not entered remission (6/17). All patients entering remission, except one, had normal or increased sensitivity to both drugs compared to normal controls, and all patients who not entered remission, with one exception, had decreased sensitivity to one or both drugs compared to normal controls. Comparison of the reduction of clonogenic cells by [^3H]thymidine or ara-C suggested that in some cases ara-C killed more cells than those in S-phase, and in some cases the cells seemed to be metabolically resistant to ara-C. We conclude that in vitro drug sensitivity tests on leukemic clonogenic cells reflect the patient's in vivo response to the tested drugs and may be used to study the biological properties of leukemia cells that determine their drug sensitivity.

INTRODUCTION

Although a majority of patients with acute leukemia achieve remission with currently used chemotherapeutic regimens cytostatic drugs produce considerable toxicity also in those patients where therapy fails to induce remission. Some of these patients will eventually achieve remission after a change to another chemotherapeutic regimen but it is not known if the initial therapy alters the chances for subsequent remission. It would therefore be an improvement if the therapeutic response to chemotherapy could be predicted and the most effective regimen selected.

The leukemic cell population is dependent on an influx of cells from a leukemic stem cell pool because of the limited proliferative capacity of the majority of leukemic blast cells (1). Normal and leukemic stem cells are clonogenic in vitro and it is thus possible to study the effects of chemotherapeutic drugs on these cells in vitro. Such tests could possibly provide a means to predict the response to chemotherapy in acute leukemia and eventually be used to select the most efficient drug combination in individual patients. Another development would be to use such in vitro tests to establish the efficacy of new drugs before clinical trials are started. There are a few reports demonstrating the feasibility of this approach in leukemia. One report (2) showed that patients with acute myeloid leukemia whose clonogenic leukemic cells were sensitive to both cytosine arabinoside and daunorubicin in vitro entered remission while patients resistant to either or both drugs failed to enter remission. Similar findings have been reported by others (3), while another study (4) demonstrated that the self-renewal capacity of blast progenitor cells in peripheral blood showed a stronger correlation to induction of remission and survival than the in vitro sensitivities to cytosine arabinoside or doxorubicin. Large efforts have also been made to develop clonogenic tumor stem cell assays for quantitation of chemotherapy drug sensitivity in vitro of non-hematopoietic cells (5).

In the present report we have studied the in vitro drug sensitivity of normal and leukemic clonogenic cells in agar using cytosine arabinoside and daunorubicin over a wide concentration range and different incubation times to establish a standardized method for in vitro drug

sensitivity testing. In seventeen patients with acute myeloid leukemia the in vitro results, with two exceptions, reflected the in vivo response to therapy in terms of achievement of remission.

MATERIALS AND METHODS

Patients

Twenty-four patients with acute myeloid leukemia were studied. Four of them did not grow in vitro and were excluded from further analysis. Of the remaining twenty patients the studies were performed on bone marrow cells obtained at diagnosis in nineteen cases and in one case (no. 17) on cells obtained after five courses of TRAP; in one case (no. 13) cells both from diagnosis and after six courses of TRAP were studied. Seventeen of the patients were treated and analyzed for correlation between in vitro and in vivo response to chemotherapeutic drugs. The FAB-classification (6) was used for morphological diagnosis. The twenty patients studied for drug sensitivity are presented in table I.

Treatment

Induction therapy (TRAP) consisted of daunorubicin 60 mg/m^2 on day 1, ara-C 70 mg/m^2 twice daily i.v. in bolus dose on days 2-6, 6-TG 70 mg/m^2 twice daily on days 2-6 and prednisone 30 mg/m^2 on days 2-6. The treatment was repeated if possible on day 10. Consolidation therapy consisted of TRAP and maintenance therapy, when given, was TRAP alternating with COAP.

Complete remission (CR) was defined as normal bone marrow cellularity with less than 5% blast cells, return of neutrophil counts to $1.5 \times 10^9/\text{liter}$ or greater, platelet counts of at least $100 \times 10^9/\text{liter}$ and hemoglobin value of at least 100 g/liter . Eleven patients achieved remission and six patients did not. Of the three remaining patients two had refractive anemia with excess of blasts (RAEB) for 3 and 20 months respectively (no. 19 and 20) prior to overt leukemia; one of these patients was treated with ara-C and thioguanine intermittently; table 1.

Cell preparation

Bone marrow cells, 3-5 ml, obtained by sternal puncture were collected in heparinized McCoy's medium, suspended by repeated suction through a needle (0.8 x 80 mm) and separated on Isopaque-Ficoll (Lymphoprep, Nyegaard & Co, Oslo). The mononuclear cell fraction was washed twice in McCoy's medium and adjusted to 3×10^6 cells per ml in McCoy's medium containing 1% FCS (Flow Laboratories, Solna, Sweden). Normal marrow cells were obtained from healthy volunteers.

Exposure to cytosine arabinoside and daunorubicin in vitro

Ara-C (Cytosar, Upjohn) and daunorubicin (Cerubidin, Leo Rhodia) were dissolved in 0.15 M NaCl, filter sterilized and diluted in McCoy's medium. Fresh solutions were made for each experiment. Fifty μ l of drug solution was added to 0.5 ml bone marrow cells, 3×10^6 /ml, to achieve final drug concentrations of 4.8, 2.4, 1.2, 0.6, 0.3, and 0.15 μ g/ml ara-C, and 0.6, 0.3, 0.15, 0.075, 0.037 and 0.018 μ g/ml daunorubicin. The cells were then incubated at 37°C for one hour in a CO₂-incubator, and washed twice in McCoy's medium 1% FCS before culture in agar. Cells incubated in medium only served as controls. In some experiments combinations of ara-C and daunorubicin were used and in other experiments the effect of 2-5 hours preincubation was studied.

Tritiated thymidine suicide

1.5×10^6 bone marrow cells in one ml McCoy's medium with 1% FCS was incubated with and without the addition of 50 μ Ci/ml [³H]thymidine (20 Ci/mmol, Amersham, England) for 30 min at 37°C. The cells were washed once in McCoy's medium containing 100 μ g/ml cold thymidine and once in medium with 1% FCS before plating in agar. The reduction of the number of clonogenic cells with [³H]thymidine was taken as a measure of the fraction of cells in S-phase (7).

Agar culture technique

Control cells and cells exposed to ara-C, daunorubicin or [³H]thymidine were cultured in agar at a concentration of 3.0×10^5 /ml in 1 ml of 0.3% agar in McCoy's medium supplemented with 15% FCS and 10% human placenta conditioned medium (8) on top of 1 ml 0.5% agar in McCoy's medium and 15% FCS in 35 mm petri dishes (Falcon Plastics, Cockeysville, MD). Four replicates were made and incubated in 37°C in 5% CO₂ in a fully humidified atmosphere. On day 10, clusters

(10-40 cells) and colonies (more than 40 cells) were counted in an inverted microscope. The patients had 34-1620 (median 167) colonies and clusters per dish and the normal controls had 119-591 (median 323).

Calculation of results

The number of surviving clonogenic cells was plotted versus drug concentration on a semilogarithmic paper. Daunorubicin gives single exponential curves from which LD_{50} -values (i.e. the concentration causing 50 per cent reduction of clonogenic cells) can be calculated from the slope of the curve by the method of least mean squares. Ara-C in most cases shows slightly biphasic curves with an initial slope corresponding to sensitive cells in DNA-synthesis and a second plateau-like phase corresponding to insensitive cells not in DNA-synthesis. LD_{50} -values for ara-C were calculated over the concentration range 0-1.2 or 2.4 $\mu\text{g/ml}$ depending on where the curve turned from the initial slope to the plateau phase.

In addition, we plotted the data in linear box diagrams and calculated the area under the curve (AUC), which represents the fraction of surviving cells; the AUC-values shown were derived from the following formula: area under the curve (=surviving cells)/total area (=surviving plus dead cells). AUC was calculated over the concentration ranges 0-2.4 $\mu\text{g/ml}$ ara-C and 0-0.3 $\mu\text{g/ml}$ daunorubicin in the standard assay.

RESULTS

Survival of clonogenic cells after drug exposure

Figure 1 shows typical survival curves of clonogenic cells after one hour exposure to increasing concentrations of ara-C and daunorubicin. Drug resistance is demonstrated by flat curves with minimal or no reduction of clonogenic cells. Drug sensitive cells gave a single exponential curve for daunorubicin, whereas ara-C typically gave a biphasic curve because primarily cells in S-phase are killed.

We also investigated the effect of ara-C and daunorubicin as a function of time (figure 2). Both drugs, at two different concentrations, were effective within one hour and there was no major additional effect with prolonged incubation up to five hours. Further experiments in seven patients compared the survival of clonogenic

cells after one and three hours preincubation with 0.6 and 2.4 $\mu\text{g/ml}$ ara-C and 0.075 and 0.30 $\mu\text{g/ml}$ daunorubicin respectively; these experiments too showed that most of the cytotoxic effect was expressed already after one hour (data not shown). Therefore, one hour incubations were chosen for standard assays.

Combined exposure to ara-C and daunorubicin

In four patients combinations of ara-C and daunorubicin were tested, figure 3. Patient A was sensitive to both ara-C and daunorubicin and the combination of ara-C and daunorubicin caused an at least additive killing effect. Patient B was sensitive to ara-C but showed only marginal inhibition with daunorubicin; however, the combination of the two drugs caused increased killing at the highest concentration. Patients C and D had low sensitivity to both ara-C and daunorubicin and the combination did not result in any increased killing.

Comparison of LD₅₀-values and AUC

LD₅₀-values and AUC were calculated for ara-C and daunorubicin in all twenty patients. There was a strong correlation between AUC and log LD₅₀-values for both ara-C ($r=0.95$) and daunorubicin ($r=0.92$). Table 2 shows the AUC for ara-C and daunorubicin. Marrow cells from one patient (no. 13) were studied twice; at diagnosis AUC was 0.88 and 0.90 for ara-C and daunorubicin respectively and after six courses of TRAP AUC was 0.86 and 0.88.

Relationship between ara-C and tritiated thymidine exposure

Tritiated thymidine suicing is an established method for determination of the fraction of clonogenic cells in S-phase (7). Ara-C kills the cells during DNA-synthesis (9) and a correlation between the number of surviving cells after exposure to ara-C and [³H]thymidine would be expected. As shown by the data in table 2 this is not always the case. In cases 2, 7, 10, 12, 16 and 17 ara-C kills fewer cells than would be expected from the fraction of cells in S-phase, whereas in case 19, and possibly cases 3 and 4, ara-C kills more cells than expected.

Correlation between in vitro drug sensitivity and in vivo response to therapy

Figure 4 shows the relationship between in vitro drug sensitivity and the clinical outcome in the seventeen patients treated with TRAP. With regard to ara-C, patients who achieved remission showed significantly higher in vitro sensitivity (mean AUC: 0.59 ± 0.16 S.D.) than patients not achieving remission (mean AUC: 0.86 ± 0.12 S.D.), $p < 0.005$. This difference was also found for daunorubicin; patients entering remission were more sensitive to daunorubicin (mean AUC: 0.56 ± 0.17 S.D.) than those not entering remission (mean AUC: 0.78 ± 0.14 S.D.), $p < 0.02$ (Student's t-test).

In figure 5 the AUC-values for ara-C are plotted versus AUC for daunorubicin together with the normal mean values ± 2 S.D. With one exception all patients achieving remission (solid circles) had normal or increased drug sensitivity for both ara-C and daunorubicin; the exception was patient no. 6 who was insensitive to daunorubicin but extremely sensitive to ara-C. Among the patients not entering remission (open circles) all except one showed decreased in vitro sensitivity to one or both drugs. The exception was patient no. 14, who had normal sensitivity to both ara-C and daunorubicin but died after two courses of TRAP from an intracerebral bleeding. Patient no. 12 had normal sensitivity to daunorubicin but was insensitive to ara-C and died from intracerebral bleedings after two courses of TRAP. Three patients were not treated with TRAP (open triangles); one of them, patient no. 18, had normal sensitivity to daunorubicin but was insensitive to ara-C and the two patients who had RAEB prior to leukemia (patients no. 19 and 20) showed normal sensitivity for daunorubicin and increased sensitivity to ara-C.

DISCUSSION

This study describes a standardized method for in vitro drug sensitivity testing of clonogenic cells in acute myeloid leukemia and demonstrates a correlation between in vitro results and in vivo response to therapy.

The concentrations of ara-C and daunorubicin were chosen to fall within the range that can be achieved in plasma (10-14). Daunorubicin produced single negative exponential curves in agreement with earlier

findings (15, 16) which allows calculation of LD_{50} -values from the slope of the curve. Ara-C on the other hand acts primarily on cells in S-phase and produces slightly biphasic survival curves and LD_{50} -values can only be extrapolated from the first part of the curve. We found that a calculation of the area under the curve, AUC, in linear box diagrams reliably reflects the in vitro drug sensitivity for both ara-C and daunorubicin and strongly correlates to the LD_{50} -values. The construction of a dose-response relationship is preferable to the use of single drug concentrations; e.g. sampling errors at one of the drug concentrations become less important when a dose-response curve is constructed, and furthermore, as illustrated in figure 1, major differences between some patients would have passed unobserved if only single drug concentrations in the lower range had been studied.

Maximal effect of both drugs was seen after one hour incubation. Short incubation times are preferable because prolonged incubation could interfere with the cell cycle status.

We found a strong correlation between in vitro drug sensitivity and in vivo response to therapy. In general, only those patients sensitive to both ara-C and daunorubicin entered remission. However, exceptions to this general agreement between in vitro sensitivity and response to therapy emphasize the fact that other factors than drug sensitivity contribute to the clinical outcome.

These findings agree with previous reports (2, 3). In one study (2) the cells were incubated with 0.3 and 3.0 $\mu\text{g/ml}$ ara-C and 0.1 $\mu\text{g/ml}$ daunorubicin for one hour prior to culture in agar. By relating the fraction of surviving clonogenic cells at 0.3 $\mu\text{g/ml}$ ara-C and 0.1 $\mu\text{g/ml}$ daunorubicin to the in vivo response the author found that patients entering remission had more sensitive cells than those who failed to enter remission. Only those patients who were sensitive to both drugs responded favorably to treatment. It was also found that simultaneous exposure to ara-C and daunorubicin was the most sensitive test for recognition of patients who responded with remission or no remission to therapy (2). In four cases tested, we found that combination of ara-C and daunorubicin at suboptimal concentrations resulted in an increased killing provided that the cells were sensitive to at least one of the drugs tested alone. Studies of the

combined effects of chemotherapeutic drugs in vitro deserve further exploration since they point to the possibility to find the most effective combination of drugs that eventually could be used in vivo.

In another study (3) leukemia cells from nine patients with AML were exposed to single drug doses of vincristine, ara-C, prednisone, rubidazole, 6-TG and doxorubicin and the results compared with those of a simultaneously studied normal control. Patients who had cells more sensitive than the control entered remission, whereas patients with less sensitive cells did not. However, since there was a considerable variation in sensitivity among the controls the outcome of the test will depend very much on the sensitivity of the randomly chosen control.

In agreement with a previous report (2) a comparison of the suicidal effect of tritiated thymidine and ara-C suggested that in some cases ara-C killed more cells than those in S-phase and in other cases clonogenic cells in S-phase seemed to be metabolically resistant to ara-C. The mechanisms on a cellular level for these discrepancies may be related to the degree of retention of ara-C in the leukemic cells (17) and may be of critical importance for the in vivo response to ara-C.

In studies of cytostatic drug effects on blast progenitor cells in peripheral blood sensitivity to ara-C but not to doxorubicin correlated to the clinical outcome (4). However, only self-renewal capacity of the blast progenitor cells was related to survival; a low self-renewal capacity was prognostically favorable (4, 18). It was also reported that the in vitro drug sensitivities to ara-C and doxorubicin and the self-renewal capacity are heritable properties within the blast progenitor cell population and change only slowly (19). We observed one patient (no. 13) who had identical sensitivity to both ara-C and daunorubicin at diagnosis and after six courses of TRAP. However, some patients may change their sensitivity during the course of the disease (2, 19).

Although it is possible to demonstrate a correlation between in vitro sensitivity to chemotherapeutic drugs and the response to therapy it is too early to apply the tests for selection of the most effective therapy on an individual basis. As pointed out the drug sensitivity is

only one of several factors that determine the clinical response to therapy. At present we regard it as more important to develop the in vitro tests for studies of the biological properties of the leukemic cells that determine their drug sensitivity. As a long term goal it should be possible to use the in vitro tests for development of new chemotherapeutic regimens and perhaps to design individualized chemotherapy.

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Patient	Sex/Age	Diagnosis (FAB)	No. of TRAP- (a) courses	CR/NR	Duration of 1st CR (months)	Survival (months)
1.	F/19	M5	3	CR	4+	5.8+
2.	F/25	M1	3	CR	7.3	20+
3.	F/41	M2	3	CR	5.4	14+
4.	F/43	M2	3	CR	27+	29+
5.	M/45	M1	3	CR	6.0	10.5
6.	M/53	M4	3	CR	5.9	12
7.	M/55	M1	5	CR	2.5	7
8.	F/56	M2	1	CR	5.6+	6.5+
9.	F/57	M3	2	CR	3.7	5.1
10.	F/58	M1	2	CR	7.8	13.9
11.	F/59	M2	2	CR	3.1	8.9
12.	F/37	M3	2	NR	-	1.5
13.	M/54	M4	7	NR	-	6
14.	M/57	M4	2	NR	-	1
15.	F/63	M2	2	NR	-	1.9
16.	F/65	M4	3	NR	-	4
17.	M/66	M2	9	NR	-	8
18.	F/77	M4	not treated	-	-	0.5
19.	F/85	RAEB - M2	"	-	-	0.2
20.	F/57	RAEB - M4	ara-C + 6-TG	NR	-	9.5

(a), the number of TRAP courses to achieve remission in CR-patients, and the total number of TRAP courses in NR-patients.

TABLE 2.

The surviving fraction of clonogenic cells after in vitro exposure to [^3H]thymidine, ara-C or daunorubicin.

Patient	[^3H]thymidine	ara-C	daunorubicin	CR/NR
1.	-	0.76	0.38	CR
2.	0.22	0.68	0.59	CR
3.	0.60	0.44	0.48	CR
4.	0.75	0.59	0.40	CR
5.	0.68	0.58	0.61	CR
6.	-	0.21	0.92	CR
7.	0.27	0.76	0.68	CR
8.	-	0.71	0.62	CR
9.	0.45	0.54	0.24	CR
10.	0.31	0.72	0.67	CR
11.	-	0.50	0.54	CR
12.	0.33	0.97	0.52	NR
13.	-	0.88	0.90	NR
14.	-	0.62	0.70	NR
15.	-	0.83	0.94	NR
16.	0.60	0.97	0.83	NR
17.	0.44	0.88	0.78	NR
18.	-	0.93	0.56	-
19.	0.70	0.36	0.50	-
20.	0.39	0.31	0.62	-
Normals	0.63	0.69	0.56	
(mean $\pm$ S.D.)	$\pm$ 0.04	$\pm$ 0.08	$\pm$ 0.10	
	(n=14)	(n=8)	(n=8)	

The data for [^3H]thymidine suiciding was calculated from exposure to a single dose of [^3H]thymidine, 50 uCi/ml, and the data for ara-C and daunorubicin are the AUC-values over the concentration ranges 0-2.4 ug/ml ara-C and 0-0.3 ug/ml daunorubicin.

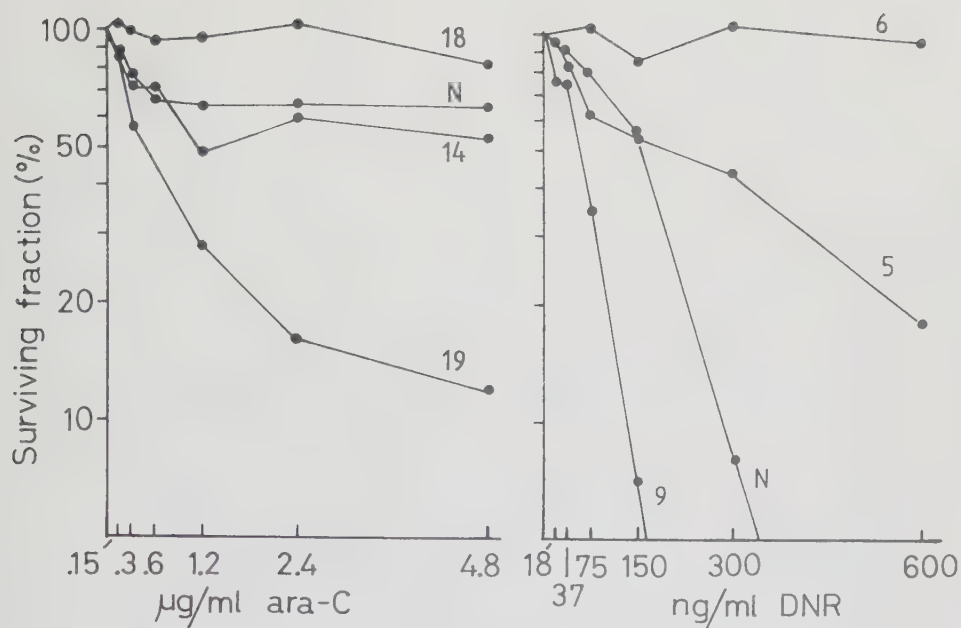


Figure 1. Survival curves for clonogenic cells after one hour exposure to increasing concentrations of ara-C and daunorubicin. The numbers in the figure refer to the patient numbers in table 1. N, normal control; DNR, daunorubicin.

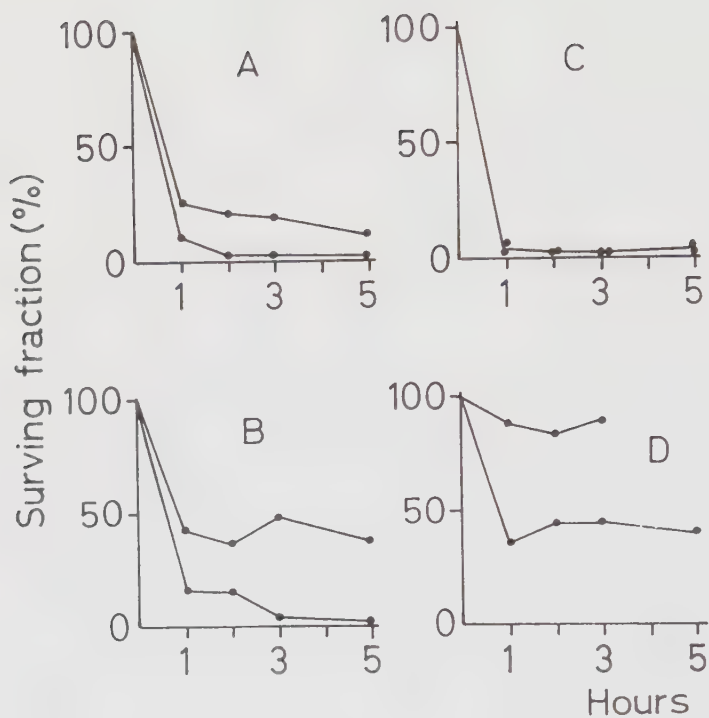


Figure 2. The effect of different preincubation times with ara-C and daunorubicin on the number of surviving clonogenic cells. Each panel shows two different patients. A: 4.8 ug/ml ara-C; B: 1.2 ug/ml ara-C; C: 0.3 ug/ml daunorubicin; D: 0.075 ug/ml daunorubicin.

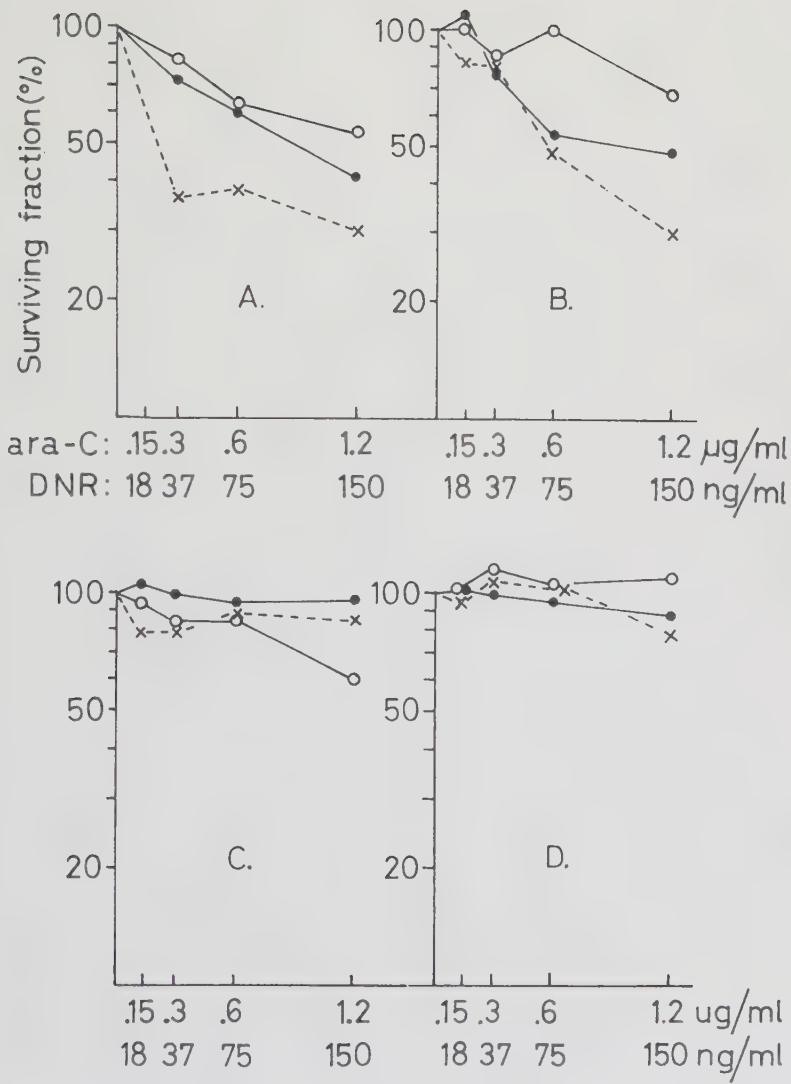


Figure 3. Survival curves for clonogenic cells after one hour exposure to ara-C or daunorubicin alone, or in combination. Four different patients are shown. (●—●), ara-C; (○—○), daunorubicin; (X—X), ara-C plus daunorubicin. DNR, daunorubicin.

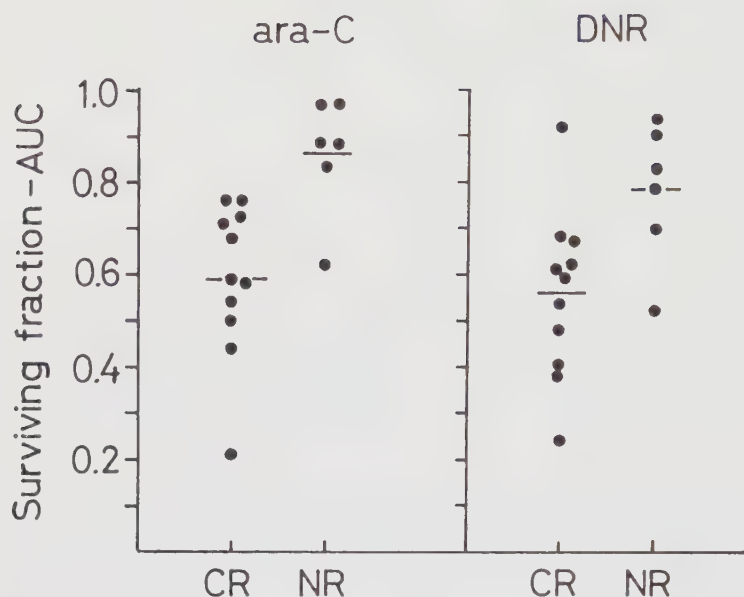


Figure 4. Comparison of in vitro drug sensitivity to ara-C and daunorubicin between patients entering remission, CR, and patients not entering remission, NR. Drug sensitivity is shown as the fraction of surviving clonogenic cells calculated as AUC. The mean values are: ara-C/CR 0.59 ± 0.16 (S.D.) and ara-C/NR 0.86 ± 0.12 , $p < 0.005$; daunorubicin/CR 0.56 ± 0.17 and daunorubicin/NR 0.78 ± 0.14 , $p < 0.02$ (Student's t-test). The normal mean values were: ara-C, 0.69 ± 0.08 and daunorubicin, 0.56 ± 0.10 . DNR, daunorubicin.

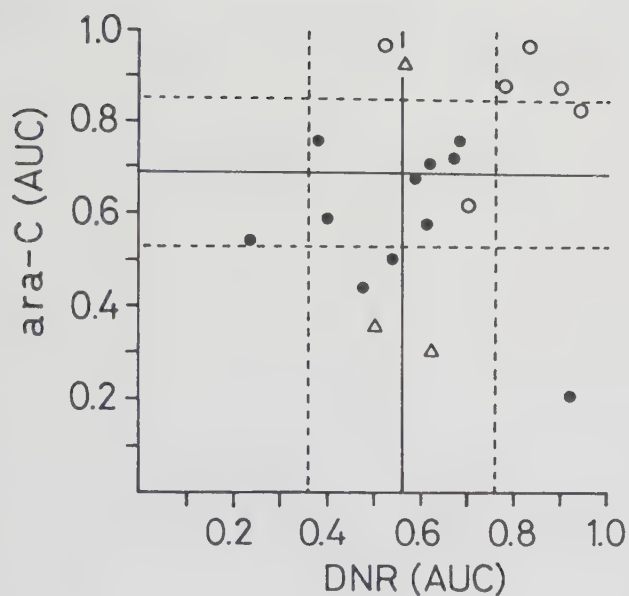


Figure 5. Correlation between in vitro sensitivity of clonogenic cells to ara-C and daunorubicin in twenty patients with AML. The horizontal lines show the normal mean value ± 2 S.D. for ara-C, and the vertical lines show the normal mean value ± 2 S.D. for daunorubicin. (●), patients who entered remission; (○), patients who not entered remission; (Δ), untreated patients (see table 1 and 2). DNR, daunorubicin.



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Oscillations of Marrow Culture Growth in Acute Myeloid Leukemia during Remission Induction and Remission

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Key Words. Acute myeloid leukemia · *In vitro* culture · Sequential studies

Abstract. Sequential studies in acute myeloid leukemia of bone marrow cells in agar culture showed striking fluctuations of colony and cluster formation during induction of remission. These oscillations may be initiated by recruitment of resting leukemic and normal cells upon chemotherapeutic perturbation. Striking oscillations of the colony and cluster formation occurred also during remission with or without maintenance therapy, but with a longer periodicity as compared to the phase of induction of remission. Oscillations of the marrow growth capacity during remission may reflect potential relapses where the outcome depends on proliferative advantages of the normal or leukemic cell clone.

Introduction

In acute myeloid leukemia (AML) profound abnormalities of the *in vitro* growth pattern of marrow cells in semisolid agar [2, 7] have been revealed [4, 8, 9, 13]. Frequently there is an absence of colonies with greater than 40 cells (CFU-C), but production of various amounts of clusters with 3–40 cells. Reclassification of AML according to *in vitro* growth pattern may have prognostic value [10]. This work presents sequential studies of the *in vitro* growth pattern in agar of marrow cells from individual AML patients undergoing pulse chemotherapy for induction of remission and for maintenance of remission. The results may

be of value in understanding how remission is achieved and maintained.

Materials and Methods

Patient Selection and Treatment

Serial studies were performed during induction of remission in 8 patients with adult AML, diagnosed using the criteria of Hayhoe and Cawley [6]. For induction of remission 5-day courses of thioguanine (100 mg/m²), cytosine arabinoside (100 mg/m²) and prednisone (30 mg/m²) were administered every 10–14 days with rubidomycin (40 mg/m²) on the first day of each course (TRAP). TRAP alternating with cyclophosphamide (100 mg/m²), cytosine arabinoside (100 mg/m²) and prednisone (200 mg/day) for 5 days with vincristine (2 mg) on the first day (COAP) was

used as maintenance therapy. Complete remission was defined as normal bone marrow cellularity with less than 5% blasts when the neutrophil counts had risen to 1,500/ μ l or greater and the platelet counts to at least 100,000/ μ l blood.

Collection of Bone Marrow

2-3 ml of bone marrow was aspirated from sternum prior to each chemotherapy course or more frequently, and collected in 3 ml of McCoy's medium, 100 IU of heparin and 75 U of Varidase (Lederle Laboratories, Pearl River, N.Y.). Marrow particles were disrupted by repeated suction

through a needle. Nucleated cells were counted in a Bürker chamber for determination of marrow cellularity.

Cell Separation

7 ml of marrow suspended in culture medium were layered on top of 5 ml Isopaque-Ficoll [1] solution in 16-ml plastic tubes. The tubes were centrifuged with a swing out rotor for 10 min at 70 g and for 15 min at 700 g. The cells collected on top of the Isopaque-Ficoll (A cells) were removed, washed three times in culture medium and used for agar culture. A cells from normal peri-

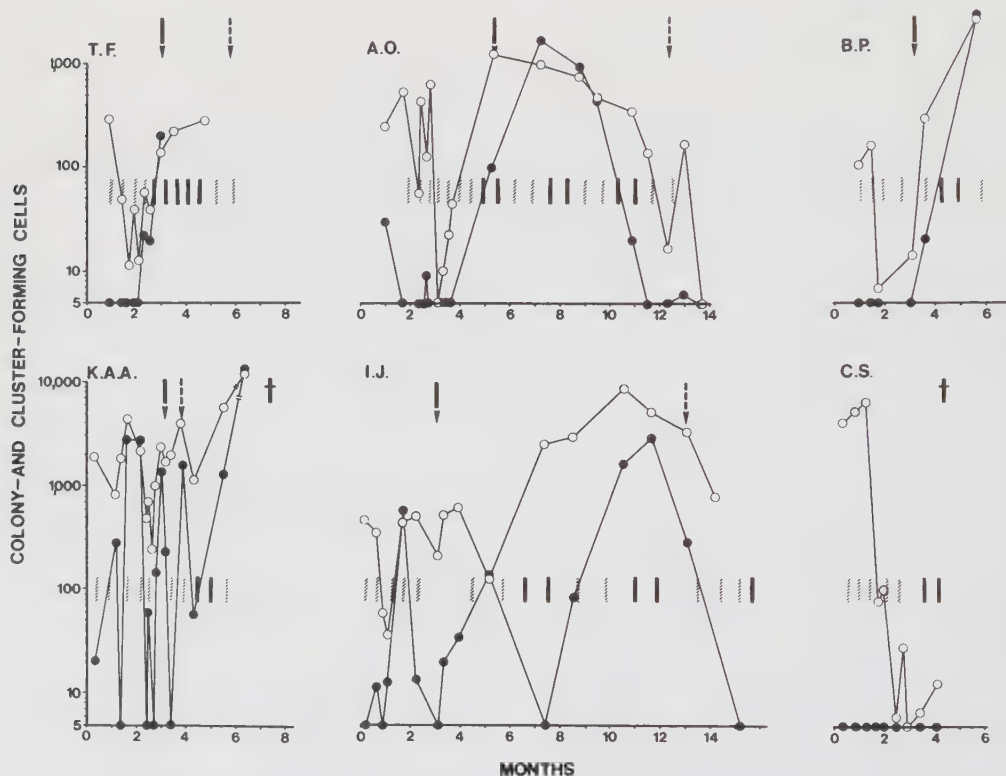


Fig. 1. Serial studies in 6 patients with AML (K.A.A., 24-year-old male; I.J., 40-year-old female; C.S., 41-year-old female; T.F., 26-year-old male; A.O., 41-year-old male; B.P., 44-year-old male) of colony-forming cells (●) per 10^6 and cluster-forming cells (O) per 10^5 seeded marrow cells. The number of cell aggregates grown is given on a logarithmic scale. The abscissa shows the

time in months after diagnosis. Chemotherapy courses are shown; TRAP (▨) and COAP (■). Complete remission (↓) (that of K.A.A. was, however, only a partial remission) and relapse (↑) are indicated. The karyotype of marrow cells from C.S. was 45 XO (8:21) and that of T.F. showed a Ph¹-chromosome t (9:22); the karyotype of the other patients was normal.

pheral blood were used for preparation of feeder layers. Colony- and cluster-forming cells were quantitatively recovered (98%) in the A cell fraction.

Cell Culture Technique

The culture technique of *Pike and Robinson* [14] was utilized. The feeder layers contained 3×10^5 A cells obtained from the peripheral blood. 1 ml of cells in 0.5% agar (Difco Bacto Agar) in culture medium was plated in 35-mm Falcon plastic Petri dishes. The over layers contained 10^5 marrow A cells from the AML patients. After gelling at room temperature the plates were incubated at 37 °C in a fully humidified atmosphere with 7.5% CO₂ in air.

Culture Scoring

Cultures were evaluated at 7 and 10 days for clusters of 3–40 cells and colonies of greater than

40 cells. Data presented for colony formation is from 10 days of culture while data for cluster formation is from 7 days of culture. On the basis of cell counts and differentials of the marrow aspirate and the A cell fraction used for plating, colony and cluster formation per microliter bone marrow were calculated (fig. 2). This was done as described previously [12].

Results

Figure 1 shows that a short phase of low or minimal growth of the marrow cells in agar is usually seen prior to complete remission (also seen in figures 2–3). However, 1 patient (C. S.) developed a nongrowing leukemia after 3 courses of TRAP, but died 3

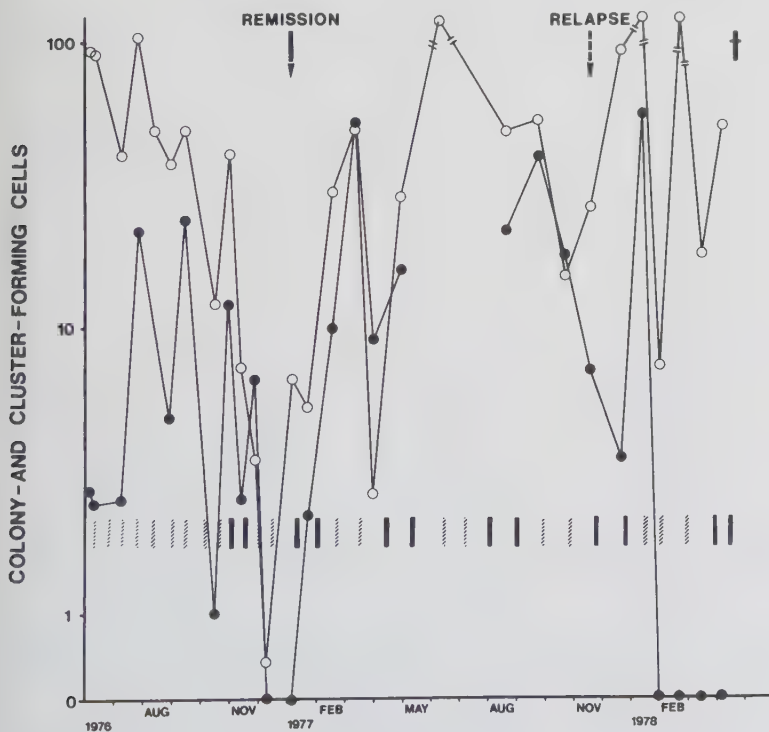


Fig. 2. Serial studies on a 46-year-old male (S.S.) with AML. The data are given as colony formation (●) and cluster formation ($\times 10^{-1}$)

(O) per μ l bone marrow. Chemotherapy courses are shown; TRAP (▨) and COAP (■). The karyotype of marrow cells was normal.

months later without achieving remission. Several patients demonstrate more or less regular fluctuations of the growth pattern during induction of remission. Colony and cluster formation of K.A.A. oscillated in a parallel fashion most of the time. The oscillations showed some correlation with the courses of chemotherapy given since both peaks and troughs usually coincided with the 5-day chemotherapy. However, an oscillation was also seen in this patient during month 3 when the interval between the TRAP courses was prolonged due to a severe infection. K.A.A. showed an extremely high colony count during relapse.

During the long induction of remission

period of the patient depicted in figure 2, pronounced fluctuations of the colony and cluster incidence were found with peaks approximately every 40–50 days. In this figure results are expressed per microliter marrow and the number of colony and cluster forming cells are seen to decrease in an oscillating fashion during induction of remission. A similar pattern was obvious when the results were expressed as relative numbers per seeded marrow cells (data not shown). However, during the relapse period some discrepancies are present since peaks and troughs do not correspond completely when data given as relative and absolute numbers are compared. This indicates that variations

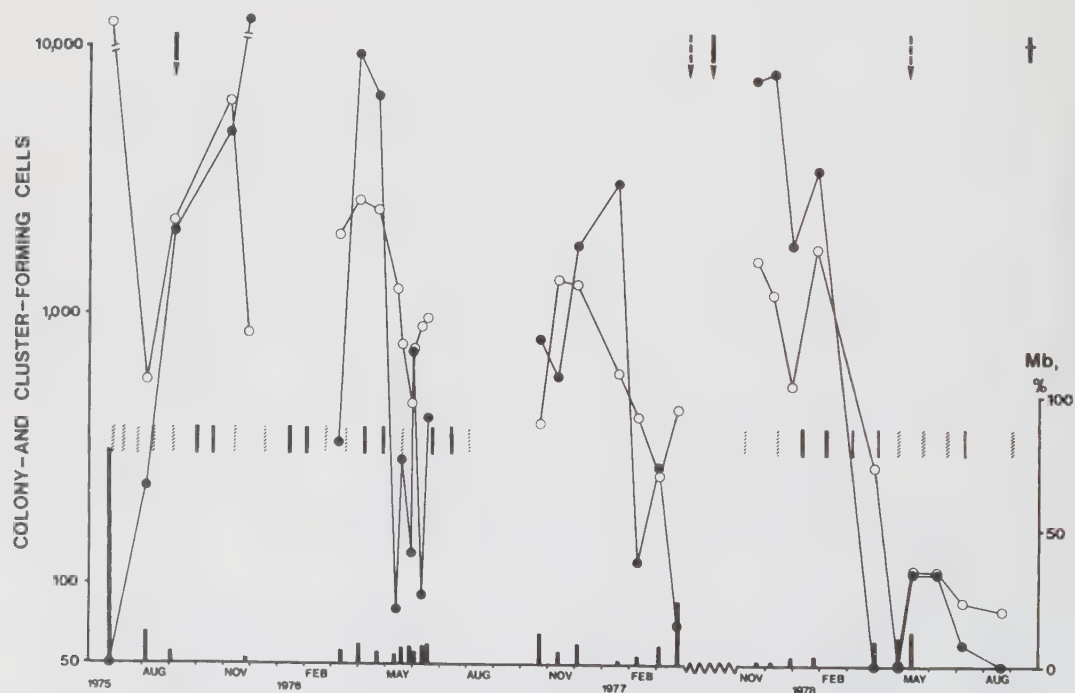


Fig. 3. Serial studies on a 44-year-old male (L.S.) with AML of colony-forming cells (●) per 10^6 and cluster-forming cells (○) per 10^5 seeded marrow cells. Data for myeloblasts of marrow are

also given. Complete remission (↓) and relapse (↑) are indicated as well as courses of chemotherapy; TRAP (▨) and COAP (■).

in relative numbers do not always coincide with variations of absolute numbers of cluster and colony forming cells.

In L. S. (fig. 3) serial analyses were carried out during long periods of the first and second remission phases. Fluctuations of the colony- and cluster-forming capacity were prominent with a periodicity of approximately 2–3 months, and persisted when no maintenance therapy was given. That wide fluctuations of the *in vitro* growth pattern can occur during remission is also demonstrated in figures 1 and 2.

The growth pattern of relapse was variable. It was usually associated with disappearance of colony formation, but sometimes with persisting fluctuations of the cluster formation. Independent of the growth pattern during relapse this stage was preceded by decreasing colony- and cluster-forming capacity sometimes to very low levels (A. O. in figure 1, S. S. in figure 2, L. S. in figure 3 at first and second relapse).

Discussion

The finding of this work is that chemotherapy induces striking oscillations of colony and cluster formation in AML both during induction of remission and remission. The interpretation of our data is complicated because agar culture does not easily discriminate between normal and leukemic colony- and cluster-forming cells. However, the initial growth pattern was characteristically leukemic in all patients studied.

We assume that oscillations of the number of colony- and cluster-forming cells are due to recruitment into active proliferative phase of resting leukemic and normal cells upon chemotherapeutic perturbation. It is

possible that leukemic blasts somehow inhibit normal clonogenic cells. In fact extracts and conditioned medium of cells from patients with acute leukemia exhibit inhibitory activity on colony formation of normal CFU-C [3]. Chemotherapy may provide the necessary decrease of blast cells to allow recruitment of normal granulopoietic precursors and periodical return of normal colony growth. The oscillations depict the struggle between leukemic and normal cells and can all be regarded as potential remissions.

Remission often seems to occur after a period of minimal growth but a prolonged period of minimal or no growth *in vitro* may be a bad prognostic feature as drug-induced aplasia is reported to be prolonged and fatal without early return of colony formation [15].

An intriguing question is whether the myeloblast population in AML requires influx from another compartment to account for its expansion. The cells which grow in agar could be particularly sensitive to chemotherapy as they have a high fraction in S-phase during induction of remission [5]. Their eradication may lead to a decrease of leukemic myeloblasts and hypothetically to recruitment of normal precursors and eventually remission.

Striking fluctuations of the colony and cluster incidence were observed also during prolonged remission in accordance with previous reports [11, 16]. The periodicity is increased during this phase as compared to the period of induction of remission, which could be due to the maintenance therapy consisting of chemotherapy administered with longer intervals. Surprisingly, however, striking fluctuations also occurred when no maintenance was given. The observation that relapse seems to occur at the initiation

phase of an oscillation could be of importance. Oscillations during remission with or without maintenance therapy may depend on competition between normal and leukemic cells, where the outcome depends on proliferative advantages of the normal or leukemic cell population.

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RELATIONSHIP BETWEEN MYELOBLASTS AND CLUSTER-FORMING CELLS IN ACUTE
NON LYMPHOCYTIC LEUKEMIA

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SUMMARY

The relationship between myeloblast-like cells and cluster-forming cells detected by growth in agar has been studied in 22 untreated patients with acute non lymphocytic leukemia by means of comparing data for ^3H -TdR labeling of myeloblast-like cells and ^3H -TdR suiciding of cluster-forming cells. The fraction of myeloblast-like cells in S-phase was significantly smaller ($13.1 \pm 8.7\%$) than the fraction of cluster-forming cells in S-phase ($35.5 \pm 15.3\%$). In 2/22 patients approximately half of the myeloblast-like cells in DNA-synthesis were calculated to produce clusters in agar which may suggest similarity for cell cycle active myeloblast-like cells and cluster-forming cells. The other patients showed decreasing plating efficiency for myeloblast-like cells in S-phase indicating non-identity or modification of the growth pattern due to environmental factors and variations in death rate during culture. Sequential studies in three patients indicated changing relationship between myeloblast-like cells and cluster-forming cells suggesting non-identity or differences in growth regulation during the course of the disease.

Key words: ANLL, ^3H -TdR labeling and suicide indices

Running title: Cell kinetics in ANLL

INTRODUCTION

It is likely that only a subpopulation of cells has extensive proliferative capacity in leukemia and displays self-renewal capacity (Buick, 1980). The response to therapy of such cells may be critical in treatment of leukemia. Stem cells committed for granulopoiesis and monocytopenoiesis (CFU-GM) give rise to colonies when grown in agar (Bradley and Metcalf, 1966; Ichikawa et al. 1966). In acute leukemia the growth pattern in agar of marrow cells is abnormal often with no colony formation but growth of various numbers of clusters (Greenberg et al., 1971; Moore et al., 1973a, 1974) which by cytogenetic analysis have been found to be of leukemic origin (Duttera et al., 1972; Moore and Metcalf, 1973b; Trent et al., 1983). It is possible that myeloblast-like cells in acute non-lymphocytic leukemia (ANLL) are maintained by a cell population detected by agar culture as cluster and colony forming cells.

Data from ^3H -thymidine labeling have shown that only a portion of the leukemic myeloblast-like cells is within the mitotic cycle, the remainder being quiescent (G_0) cells (Stryckmans et al., 1970). The clonogenic cells in leukemia detected in agar may be included in the fraction of cycling myeloblast-like cells or represent cells recruited from the fraction of quiescent myeloblast-like cells.

The present work is an attempt to study the relationship between myeloblast-like cells and cluster-forming cells in leukemia. For this purpose we have compared the fraction of cells in S-phase using ^3H -TdR labeling for myeloblast-like cells and ^3H -TdR suicing for cluster-forming cells.

MATERIAL AND METHODS

Bone marrow specimens collected from 22 patients with ANLL at the time of diagnosis were investigated. Classification was according to the criteria suggested by the French-American-British (FAB) co-operative study group (Bennett et al., 1976). In 3 patients serial studies were performed during the course of the disease on labeling and suicide indices. Studies were also made on the promyelocytic leukemic cell line HL-60 (Collins et al., 1977) in order to compare the results of the two techniques for determination of the S-phase fraction.

In vitro culture technique

The method used for culture of bone marrow cells in agar has been described in detail elsewhere (Gustavsson et al., 1981). Briefly, bone marrow cells were collected in heparinized McCoy's medium, separated in Isopaque-Ficoll (1.077 g/ml) and the cells from the interphase cultured at 1×10^5 cells/ml in 0.3% agar on top of a feeder layer containing 3×10^5 blood mononuclear cells from a single donor. Clusters (3-40 cells) and colonies (>40 cells) were scored on day 7 of incubation at 37°C in 5% CO_2 in humidified air. HL-60 cells in logarithmic growth phase were cultured at 5×10^3 cells/ml in 0.3% agar on top of 1 ml 0.5% agar with 15% fetal calf serum in both layers. Cell aggregates containing more than 3 cells were counted on day 7.

Tritiated thymidine suicide and labeling index

For determination of the proportion of progenitor cells in DNA-synthesis the ^3H -thymidine suicide technique was used. One and a half ml cells lighter than 1.077 g/ml in McCoys medium, $3\text{-}5 \times 10^6$ cells/ml, were incubated with 75 uCi of ^3H -thymidine with a specific activity of 19-21 Ci/mmol (Radiochemical Centre, Amersham, England). A control was run simultaneously without ^3H -thymidine. After 30 min of incubation at 37°C the cells were washed once in 15 ml of McCoys medium containing 100 ug/ml cold thymidine and once in medium without thymidine and then used for agar culture as described. The reduction of cluster-colony formation upon suiciding is taken as a measure of the fraction cluster/colony forming cells in DNA-synthesis: SI_{C1} . The cells suspensions incubated with ^3H -thymidine were also used for determination of ^3H -thymidine labeling index of myeloblasts by autoradiography: LI_{Mb} , using emulsion K2 (Ilford Ltd., Ilford, England). The smears were exposed for one week at 4°C and stained with May-Grünwald-Giemsa. Cells with 5 grains or more were considered labeled; 400 cells were counted.

RESULTS

Table 1 presents basic clinical data on the 22 patients and results from ^3H -TdR labeling and ^3H -TdR suiciding. Myeloblast-like cells predominated in the cell samples. The growth pattern in agar was typical of ANLL with low or absent colony formation (no patient had more than 4 colonies/ 10^5 cells) but growth of a various number of clusters ($13\text{-}3983/10^5$ cells).

The fraction of myeloblast-like cells in DNA-synthesis; LI_{Mb} , (mean $13.1 \pm 8.7\%$) was usually smaller than the fraction of cluster-forming cells in DNA-synthesis; SI_{Cl} , (mean $35.5 \pm 15.3\%$) ($p < 0.001$, t-test). In an attempt to further analyze the relationship between cluster-forming cells and myeloblast-like cells a comparison was made between the total number of clonogenic cells in S-phase and the total number of myeloblast-like cells in S-phase (table 1). This comparison is possible to make because the number of myeloblasts in S-phase can be calculated from 3H -TdR labeling indices and the number of myeloblasts/ 10^5 cells, and the number of clonogenic cells in S-phase can be calculated from 3H -TdR suicing and the number of cluster-forming cells/ 10^5 cells. It is emphasized that the two measurements are made on the same cell specimens (see Methods) and therefore the clonogenic cells in S-phase are to be found among the labeled cells identified on the smears prepared for 3H -TdR labeling indices. Thus, a ratio of one for SI_{Cl}/LI_{Mb} would mean that all myeloblast-like cells in S-phase form clones in agar (provided that the myeloblast-like cell is the predominant cell type present, which proliferates in agar). It implies that clonogenic cells are to be found among cells with myeloblast-like morphology but not necessarily being identical with the bulk of myeloblasts, i.e. the myeloblast-like cells may be heterogeneous. The number of clonogenic cells in DNA-synthesis was in all cases lower than the number of myeloblast-like cells in DNA-synthesis, the ratio SI_{Cl}/LI_{Mb} varied between 0.53-0.0006. The very high ratio found in two patients; 0.53 and 0.45, suggested that a majority of myeloblast-like cells in DNA-synthesis produced clusters in agar.

The plating efficiency for HL-60 cells was 30-35%, the LI was 30-35% and the SI was 29-30%. This means that 30-35% (i.e. plating efficiency) of the number of labeled HL-60 cells should be expected to form clones in agar. When comparing the number of clonogenic cells in S-phase with the number of 3H -TdR labeled cells we found that about a quarter of cells in DNA-synthesis produced clones in agar. Thus, there seemed to be a consistency between the two techniques when used on HL-60 cells.

Figure 1 shows sequential studies in case no.2, 3 and 20 comparing kinetics for clonogenic cells and myeloblast-like cells during remission induction, remission and relapse. During the period of induction of remission fluctuations were found both for the S-phase fraction of clonogenic cells and myeloblasts. However, the fluctuations were not identical for these cell types, in many instances they seemed to be inverse. Non-parallel oscillations were also seen during remission and relapse.

DISCUSSION

In this work we have found a higher $^3\text{H-TdR SI}_{\text{C1}}$ of clonogenic cells in agar than LI_{Mb} of the myeloblast-like cells of ANLL. The suicide index of the leukemic marrow cells was comparable with those reported for CFU-GM of normal bone marrow cells (Greenberg and Schrier, 1973; Moore et al., 1973a; Olofsson and Olsson, 1976; Liu et al., 1979). Previous studies of $^3\text{H-TdR}$ suicide indices of cluster-forming cells in ANLL reported a lower SI: $17 \pm 10\%$ (Moore et al., 1973a) or a higher: $54 \pm 8\%$ (Preisler and Shoham, 1978). Discrepancies may be explained by relatively small and heterogeneous patient materials, technical differences as well as inclusions of blood specimens in one study (Moore et al., 1973a).

The labeling index of myeloblast-like cells in our study is in accordance with previous reports (Pileri et al., 1967; Burke et al., 1977; Hart et al., 1977; Lange Wantzin, 1980; Stryckmans et al., 1980; Hiddemann et al., 1982) and considerably lower than in normal myeloblasts (Gavosto et al., 1960). Low labeling indices result from the presence of quiescent myeloblasts (Stryckmans et al., 1970).

Our findings indicate a similarity between cycling myeloblast-like cells and cluster-forming cells in two patients. The other patients, however, showed low plating efficiencies for the myeloblast-like cells in S-phase which may indicate non-identity. Even if the comparison between morphologically identified myeloblasts and cells forming clusters in agar was done with different technical means we assume that both $^3\text{H-TdR}$ labeling and $^3\text{H-TdR}$ -suiciding give reasonably accurate and comparable results for the number of cells in S-phase. This assumption is supported by the finding of almost identical LI and SI of the HL-60 cell line. Therefore it should be possible to determi-

ne if the number of myeloblast-like cells in S-phase is equivalent to the number of cluster-forming cells in S-phase, in which case identity is likely.

It is possible that cluster-forming cells maintain the population of myeloblast-like cells. Another indication of maintenance of leukemic blast cells by the proliferative activity in vitro of a small population of blast cells has been reported (Minden et al., 1978); precursors of blast cell colonies in ANLL (stimulated by PHA-LCM) were found to have a high S-phase fraction.

Sequential studies during the course of the disease showed fluctuations of the S-phase fraction of cluster-forming cells and myeloblast-like cells. Fluctuations in the fraction of myeloblast-like cells in S-phase did not, however, coincide with the variations of cluster-forming cells in S-phase indicating that myeloblast-like cells and cluster-forming cells behave differently to chemotherapy which may be due to differences in growth regulation during the course of the disease.

Thus, in only a small minority of patients do the results suggest a similarity between myeloblast-like cells in S-phase and cluster-forming cells in S-phase, and only in these cases would the results support the concept that leukemic blast cells (cluster-forming cells) may be considered to be a sub-population of blast cells with a proliferative potential. In the majority of patients the results do not suggest this relationship, though it is possible that this is a false negative result due to modifications of growth patterns by environmental factors and variations in death rate during culture.

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Table 1. Clinical data and results from ³H-TdR labeling and suiciding on 22 ANLL-patients.

Case	Sex/Age	Diagnosis	% Mb	LI _{Mb} (%)	Mb in S- phase/10 ⁵	Cl/10 ⁵	SI _{Cl} (%)	Cl in S- phase/10 ⁵	Cl in S Mb in S	CR/NR
1	F/41	M 2	67	5	3 363	3 985	45	1 782	0.53	NR
2	M/46	M 6	35	3.5	1 243	1 477	38	559	0.45	CR
3	M/41	M 1	78	2	1 560	523	37	193	0.12	CR
4	F/58	M 2	97	2.8	2 716	437	69	303	0.11	CR
5	F/50	M 2	70	7.5	5 288	1 495	22	332	0.06	NR
6	F/40	M 2	80	4	3 220	476	23	111	0.035	CR
7	F/53	M 2	57	12	6 792	984	22	221	0.033	NR
8	M/54	M 3	82	21	17 178	871	39	336	0.020	no therapy
9	F/74	M 4	83	14.5	12 006	584	37	218	0.018	" "
10	F/26	M 6	65	14	9 170	575	26	152	0.017	CR
11	M/59	M 4	51	11	5 610	135	54	73	0.013	CR
12	F/68	M 4	75	24.5	18 375	2 477	8	195	0.011	NR
13	M/82	M 2	65	4	2 620	39	56	22	0.008	no therapy
14	F/61	M 1	72	34.8	25 020	292	58	168	0.007	CR
15	M/38	M 2	77	7	5 418	92	37	34	0.006	CR
16	F/70	M -	56	18	10 080	138	37	51	0.005	no therapy
17	F/64	M 2	86	20	17 250	208	37	77	0.005	NR
18	M/49	M 4	89	12	10 740	98	40	39	0.004	CR
19	F/54	M 2	74	9.2	6 808	42	48	20	0.003	no therapy
20	M/26	M 4	87	13.5	11 779	298	13	38	0.003	CR
21	F/63	M 2	49	29	14 297	144	12	17	0.001	NR
22	F/53	M 6	39	18	7 020	14	28	4	0.0006	CR
Mean ± SD			13.1 ± 8.7				35.5 ± 15.3			
HL-60 cells			35.5	35	200	29 880	30	9 080		0.26
HL-60 cells			30.4	30	400	28 440	28	8 320		

Table I. Mb, myeloblast-like cells; Cl, cluster-forming cells; LI_{Mb} , 3H -TdR labeling index for myeloblast-like cells; SI_{Cl} , 3H -TdR suicing index for cluster-forming cells; Cl in S, total number of cluster-forming cells in S-phase; Mb in S, total number of myeloblast-like cells in S-phase. CR/NR, remission or no remission. The number of Mb in S-phase, the number of cluster-forming cells and the number of cluster-forming cells in S-phase are calculated per petri dish containing 10^5 seeded cells. For HL-60 cells only 5×10^3 cells were seeded per dish but data are given for 10^5 cells.

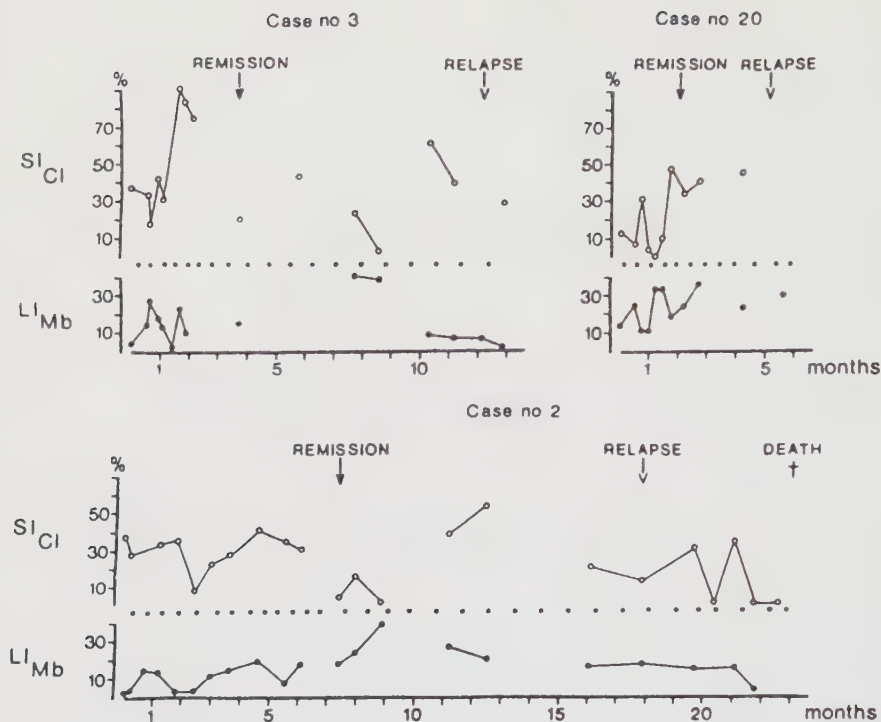


Fig.1 Sequential studies in patients 3, 20 and 2 on per cent cells in S-phase of clonogenic cells ($\bigcirc$ — $\bigcirc$) and myeloblasts ($\bullet$ — $\bullet$). Chemotherapy courses are indicated (■). The induction treatment was TRAP; cytosine-arabioside i.v., thioguanine and prednisone orally during five days with daunorubicin i.v. on the first day. The treatment was repeated if possible every tenth day. Maintenance therapy consisted of COAP (cyclophosphamide i.v., cytosine-arabioside i.v. and prednisone orally during 5 days with vincristine i.v. on the first day) twice, alternating with TRAP twice, with one cycle each month.

